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STUDIES ON THE CONTROL OF THE RENAL TUBULAR TRANSPORT OF *p*-AMINOHIPPURATE BY THE ANTERIOR PITUITARY[1]

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A REDUCTION in the renal clearance of diodrast or *p*-amino-hippurate (PAH) following hypophysectomy has been described in the dog (White, Heinbecker and Rolf, 1947, 1949a; deBodo and Sinkoff, 1953; Davis, *et al.*, 1954) and in the rat (Boss, Osborn and Renzi, 1952). These changes could be partially reversed by growth hormone (White, Heinbecker and Rolf, 1949b; deBodo and Sinkoff, 1953; Davis, *et al.*, 1954), by the thyrotrophic hormone (TSH) (Davis, *et al.*, 1954) and by adrenocorticotrophic hormone (ACTH) (Leaf, *et al.*, 1952; Earle, *et al.*, 1953). These effects of hypophysectomy could be due to changes in the renal hemodynamics and/or changes in the renal transport of these substances. Cross and Taggart (1950) have developed an *in vitro* method for measuring the transport of PAH which is independent of hemodynamic changes. Farah, Graham and Koda (1952) have shown that hypophysectomy in rats reduced PAH transport in renal slices and that the administration of certain growth hormone preparations restored this transport to nearly normal levels. The present report extends these earlier findings and the evidence presented indicates that the renal PAH transport is under the influence of a number of endocrine organs.

METHODS

Normal and hypophysectomized Sprague Dawley rats of both sexes were obtained commercially.[2] Thyroidectomy, orchidectomy or adrenalectomy of normal and hypophysectomized rats were performed in this laboratory. Animals were killed by a blow on the

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[2] Supplied by the Hormone Assay Laboratories, Chicago, Ill.

neck and the kidneys were rapidly removed, cleaned, weighed and sliced by means of a Stadie-Riggs tissue slicer. About 75–100 mg. of whole kidney slices were placed in a modified phosphate buffer devised by Cross and Taggart (1950) except that in our experiments the PAH concentration was 1.36×10^{-4} *M*. Renal slices from each animal were placed in vessels containing either no added substrate or 0.01 *M* sodium acetate. In all experiments reported here the temperature was kept constant at 25° C and the incubation time was two hours. In some of the experiments the oxygen consumption and PAH uptake were determined in the Warburg apparatus by conventional methods. In the other experiments only PAH uptake was determined by incubating the renal slices in 20 cc. beakers in a Dubnoff metabolic shaking machine. In both types of experiments the volume of buffer was 2.8 ml. and the gas phase was 100% oxygen. The accumulation of acetyl PAH by rat renal slices was also studied by substituting it for PAH in the incubation medium. The filtrates were acidified with HCl (final concentration one normal) and placed for one hour in a boiling water bath and PAH was then determined in the usual manner. Following the procedure of Cross and Taggart (1950) PAH uptake was expressed as the slice medium ratio (S:M ratio).

Growth hormone (GH)and the other pituitary hormonal preparations[3] were dissolved in slightly acid or basic saline. In all experiments the daily dosage of hormone was given in two injections intraperitoneally, one in the morning and the second in the late afternoon. In those instances where administration of the hormone preparation was continued for 5 days, the rats were killed on the morning of the 6th day and the experiment was conducted on the kidneys of these animals. Body and some wet organ weights were recorded in all of these experiments.

TABLE 1. THE EFFECT OF SEX AND WEIGHT OF NORMAL AND HYPOPHYSECTOMIZED RATS ON THE PAH S:M RATIO OF KIDNEY SLICES

	Body weight in grams	No. of rats	S:M ratio		Ratio kidney weight to body area
			No substrate added	NaAc 0.01 *M*	
Male normal	86–116	10	9.5±0.5*	14.2±0.6*	0.369
Male normal	216–260	22	9.5±0.6	14.2±0.7	0.574
Male hypox. 27–35 days post-op.	79–104	24	4.4±0.4	5.9±0.3	0.322
Male hypox. 33–35 days post-op.	231–257	12	5.0±0.3	6.1±0.4	0.512
Female normal	78– 93	7	10.1±0.8	13.7±0.6	0.347
Female normal	186–224	7	9.2±0.6	14.0±0.9	0.584
Female hypox. 31 days post-op.	84–101	7	5.1±0.4	6.2±0.3	0.302
Sham operated male 27 days post-op.	189–236	12	9.6±0.8	14.2±1.2	0.545

* In this and next tables these values indicate standard deviation.

RESULTS

Renal PAH uptake was determined in normal and hypophysectomized male and female rats and in animals of different ages. The results obtained are summarized in Table 1 and neither sex or age produced any significant variation in PAH transport. Hypophysectomy reduced this function in all the groups studied.

[3] These preparations were kindly supplied by Dr. I. M. Bunding of the Armour Laboratories, Chicago, Ill.

The reduction of PAH transport following hypophysectomy was apparent on the second and reached a maximum on the eighth to tenth postoperative day (Fig. 1). The decrease in the PAH S:M ratio was more marked in the presence of acetate than in the absence of added substrate (Fig. 1). Changes in oxygen consumption and PAH uptake of renal slices were determined in the Warburg apparatus and hypophysectomy decreased both these functions. The percent reduction in PAH uptake as well as oxygen consumption was greater with added acetate than in the absence of added substrate. The ratio of kidney weight to body surface area was also decreased by hypophysectomy (Table 2).

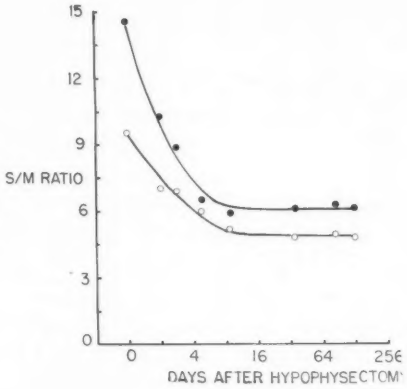

FIG. 1. The effect of time following hypophysectomy on the PAH S:M ratio of rat renal slices. (Each point represents the average of 6–12 animals.) Open circles: No added substrate. Full circles: With 0.01 *M* sodium acetate as substrate. Male animals used.

The reduction in PAH uptake following hypophysectomy could be due to an increased acetylation of PAH by renal slices and thus simulate a reduction in PAH uptake. Acid hydrolysis of the filtrates from kidney slices and medium indicated that some acetylation of PAH occurred in both control and hypophysectomized rats, however, this did not appreciably modify the values of the S:M ratio. To settle this point conclusively we determined the effects of hypophysectomy on the renal transport of acetyl PAH and the results are presented in Table 3. It is apparent that acetyl PAH transport was depressed by hypophysectomy, and growth hormone therapy (Armour, Lot R 377237) produced a recovery of this transport mechanism.

Another possibility, namely, the lack of a Krebs cycle intermediate in the kidney tissue from hypophysectomized rats was considered. The addition of α ketoglutarate, succinate or malate (0.002 *M*) to the buffer did not modify the reduction in renal PAH uptake of hypophysectomized rats.

The depressed PAH uptake and oxygen consumption of renal slices from hypophysectomized rats was increased by the administration of growth hormone (Table 2). All the GH preparations studied produced approximately equal body and kidney weight increases but the recovery of PAH

TABLE 2. PAH S:M RATIO AND OXYGEN CONSUMPTION OF RAT RENAL SLICES FROM NORMAL, HYPOPHYSECTOMIZED AND GROWTH HORMONE TREATED MALE ANIMALS. DOSAGE OF GROWTH HORMONF 0.5 MGM. 2× DAILY FOR 5 DAYS

	No. of rats	Range of body wt. in gms. initial wt.	Body wt. change (5 day period) gm.	PAH S:M ratio		O_2 consumption per mg. wet wt.		Ratio kidney weight to body surface area	TSH activity of GH prep per mg.
				No substrate added	0.01 *M* NaAc	No substrate added	0.01 *M* NaAc		
Normal (Control)	8	200–246	+24	9.3 ±0.9	13.3 ±1.2	1.476 ±0.138	1.974 ±0.110	0.562	
Sham operated 22–25 days post-op.	7	189–242	+32	9.7 ±0.7	14.0 ±1.1	1.484 ±0.14	1.980 ±0.124	0.539	
Hypophysectomized 28–31 days post-op.	20	84–105	+3.5	5.0 ±0.5	6.1 ±0.5	1.116 ±0.108	1.193 ±0.08	0.324	
Normal +growth hormone (Armour, Lot R 377237)	6	226–248	+26	9.0 ±0.6	13.8 ±0.8	1.422 ±0.12	2.041 ±0.152	0.574	0.11 units
Hypophysectomized +growth hormone (Armour, Lot R 377237)	8	87– 95	+21	8.9 ±0.7	12.6 ±0.8	1.387 ±0.117	1.908 ±0.136	0.383	0.11 units
Hypophysectomized +growth hormone (Armour, Lot K 40805R)	6	89– 97	+23	8.3 ±0.6	13.5 ±0.9	1.542 ±0.083	1.986 ±0.112	0.389	0.37 units
Hypophysectomized +growth hormone (Armour, Lot 491092B)	8	93–104	+24	8.6 ±0.6	11.8 ±1.2	—	—	0.392	contain detectable TSH
Hypophysectomized +growth hormone (Armour, Lot 369-202B)	8	86–103	+24	6.6 ±0.4	7.3 ±0.5	1.239 ±0.086	1.615 ±0.115	0.398	less than 0.02 units
Growth hormone (Armour, Lot 491082P)	8	86– 94	+21	6.4 ±0.4	7.9 ±0.6	1.278 ±0.123	1.556 ±0.090	0.386	less than 0.06 units

TABLE 3. THE RENAL S:M RATIO OF ACETYL PAH AS INFLUENCED BY HYPOPHYSECTOMY AND GROWTH HORMONE THERAPY. GROWTH HORMONE USED WAS ARMOUR LOT R 377237. MALE HYPOPHYSECTOMIZED RATS 31 TO 35 DAYS AFTER HYPOPHYSECTOMY

	No. of rats	S:M ratio	
		No substrate Added	0.01 *M* NaAc
Normal (Control)	6	9.6±0.6	12.9±0.7
Hypox.	6	3.9±0.3	5.6±0.2
Hypox+GH 0.5 mg. 2× daily for 5 days	6	9.0±0.7	12.4±0.5
Normal+GH as above	6	9.3±0.5	13.6±0.9

transport varied with different preparations. Thus GH Lot R 377237, lot K 40805R and lot 491092B produced nearly complete recovery of PAH uptake and oxygen consumption; GH lot 369202B and lot R 491082P, produced only a partial recovery of these parameters.

The time factor for producing recovery of PAH uptake was studied with GH lot R 377237. The *in vitro* addition of GH in a concentration of 0.1–0.2 mg. per ml. of buffer had no effect on the PAH S:M ratio of kidney slices from normal and hypophysectomized rats. Administration of growth hormone (0.5 mg. 2× daily) to hypophysectomized rats produced an increase in PAH uptake on the 3rd day of injection and reached its maximum on the fifth to seventh day.

The amount of growth hormone necessary to produce maximal effects was determined with GH lot R 377237 and the results are given in Table 4. It is apparent that the response of body and kidney weight to growth hormone was far more sensitive than the renal transport of PAH.

The more active GH preparations studied (see Table 2) contained thyrotrophic hormone (TSH) while the less active ones contained no detectable amounts of this hormone. This observation suggested that contaminants of GH may play a role in the recovery of PAH uptake. Table 5 contains data on a number of pituitary hormonal preparations which could conceivably influence PAH uptake. Of all the preparations studied only TSH

TABLE 4. THE EFFECT OF DOSAGE OF GROWTH HORMONE (ARMOUR, LOT R 377237) ON THE PAH S:M RATIO OF RENAL SLICES OBTAINED FROM HYPOPHYSECTOMIZED MALE RATS (SPRAGUE DAWLEY, WT. 98–116 GM.). INJECTIONS WERE GIVEN INTRAPERITONEALLY TWICE DAILY FOR 5 DAYS AND WERE STARTED ON THE 31ST–38TH DAY FOLLOWING HYPOPHYSECTOMY

	Dosage of GH mg. given 2× daily	No. of rats	Body wt. change, gms.	PAH S:M ratio	
				No substrate added	0.01 *M* NaAc
Hypox. (Control)	—	11	+ 3	4.9±0.6	6.0±0.4
Growth Hormone	0.1	7	+24	6.5±0.5	8.3±0.8
Growth Hormone	0.25	10	+26	8.3±0.5	11.4±0.5
Growth Hormone	0.5	10	+20	9.6±0.7	14.0±0.9
Growth Hormone	1.0	10	+19	9.2±0.6	13.3±0.8

TABLE 5. THE EFFECT OF SOME PITUITARY HORMONE PREPARATIONS ON THE PAH S:M RATIO OF RENAL SLICES OBTAINED FROM HYPOPHYSECTOMIZED MALE SPRAGUE DAWLEY RATS (WEIGHT 92–112 GM.). EXPERIMENTS WERE CONDUCTED 21 TO 28 DAYS AFTER HYPOPHYSECTOMY. 5 HORMONAL PREPARATIONS WERE GIVEN INTRAPERITONEALLY TWICE DAILY FOR 5 DAYS

	Dosage of hormone given 2× daily for 5 days	No. of rats	PAH S:M ratio		
			No substrate added	0.01 *M* NaAc	
Hypox. (control)		8	4.8±0.4	6.0±0.5	
Growth Hormone	0.5 mg.	6	9.1±0.9	13.7±1.2	Armour, Lot R 377237
Prolactin	4 units	6	4.7±0.4	5.6±0.6	Armour, Lot N 2203R
Pituitary L.H.	0.5 mg.	8	6.3±0.3	7.9±0.4	Armour, Lot 227–80
Melanophore expanding principle	0.05 mg.	4	4.2±0.3	5.6±0.5	Armour, Lot R 491140
Pituitrin	2 units	8	4.0±0.4	5.2±0.3	Parke, Davis & Co.
ACTH	1–2 mg.	14	5.3±0.4	6.0±0.4	Armour, Lot 146 R-53
Thyrotrophic hormone	1 unit	8	7.9±0.5	11.7±0.8	Armour, Lot 491030

and luteinizing hormone (LH) had an influence on renal PAH uptake. These findings suggested that thyroid and gonadal function could be factors in the control of renal PAH transport.

Thyroidectomy of normal rats produced a reduction of PAH uptake and this reduction was restored by thyroxin therapy (Table 6). The reduction in PAH uptake produced by thyroidectomy was qualitatively similar to that of hypophysectomy; however, the effects of PAH uptake developed more slowly (compare Table 6 and Fig. 1). During the early post-operative

TABLE 6. THE EFFECT OF THYROIDECTOMY AND THYROXIN THERAPY ON THE RENAL PAH S:M RATIO. MALE SPRAGUE DAWLEY RATS

	No. of rats	Body weight in grams	Days after Thyroidectomy	PAH S:M ratio	
				No substrate added	0.01 *M* NaAc
Control (Normal)	20	139–225	—	9.6±0.6	13.7±0.8
Thyroidectomy	8	142–199	2– 3	8.6±0.8	11.9±0.7
Thyroidectomy	9	132–209	4– 5	8.9±1.1	12.0±0.9
Thyroidectomy	11	161–183	8–10	7.8±0.7	11.3±1.2
Thyroidectomy	8	174–199	12–15	7.0±0.4	10.6±0.8
Thyroidectomy	14	172–185	18–23	6.4±0.6	6.9±0.5
Thyroidectomy	12	166–203	25–30	6.6±0.7	7.4±0.8
Thyroidectomy + thyroxin 0.5 gamma 2× daily for 5 days	10	179–201	23–27	7.2±0.6	10.4±0.9
Thyroidectomy + thyroxin 5 gamma 2× daily for 5 days	9	171–195	23–27	10.0±1.0	14.2±0.8
Normal + thyroxin 5 gamma 2× daily for 5 days	8	163–201	—	9.3±0.5	13.3±0.6

period acetate stimulation of PAH transport was still marked and only after the 18th–23rd post-thyroidectomy day was acetate stimulation reduced. Small doses of thyroxin restored PAH uptake more readily in the presence of acetate than in the absence of added substrate. Larger doses of thyroxin produced complete restoration of PAH uptake both in the presence and absence of substrate. It is likely that thyroxin produced some specific effects on the utilization of acetate for PAH transport.

The effects of thyroidectomy and thyroxin therapy on PAH transport suggested that the effects of hypophysectomy may be due to a depression of thyroid function. The administration of TSH or thyroxin to hypophysectomized rats produced a partial restoration of PAH uptake but even the largest doses of TSH or thyroxin used did not produce recovery to normal levels (Table 7). Here again small doses of TSH or thyroxin pro-

TABLE 7. THE INFLUENCE OF THYROTROPHIC HORMONE (TSH) ARMOUR, LOT 491030, AND THYROXIN ON PAH S:M RATIO AND OXYGEN CONSUMPTION OF RENAL SLICES FROM HYPOPHYSECTOMIZED MALE SPRAGUE DAWLEY RATS WEIGHING 93–100 GM. TSH AND THYROXIN GIVEN 2× DAILY FOR 5 DAYS

	No. of rats	Body weight change	PAH S:M ratio		Oxygen consumption per mg. wet wt.		Ratio kidney weight to body surface area
			No substrate added	0.01 *M* NaAc	No substrate added	0.01 *M* NaAc	
Normal (Control)	8	+24	9.3±0.9	13.3±1.2	1.476±0.138	1.974±0.110	0.517
Hypox. (Control)	7	+ 2	5.4±0.5	6.2±0.4	1.156±0.085	1.306±0.102	0.338
TSH 0.05 units	6	+ 4	5.9±0.4	7.2±0.3	1.216±0.075	1.674±0.093	0.341
TSH 0.1 units	8	− 5	8.3±0.6	12.0±0.9	—	—	0.354
TSH 1.0 units	8	− 4	7.9±0.5	11.7±0.8	1.384±0.128	1.958±0.155	0.330
Thyroxin 1.0 gamma	10	+ 2	6.2±0.5	8.5±0.7	1.265±0.086	1.704±0.133	0.347
Thyroxin 2.0 gamma	6	− 7	7.5±0.2	11.2±0.4	1.543±0.122	2.068±0.155	0.325
Thyroxin 5.0 gamma	10	− 6	8.2±0.5	11.9±0.6	1.641±0.124	2.211±0.157	0.353
Thyroxin 10.0 gamma	10	−13	7.6±0.6	11.4±0.7	1.715±0.142	2.092±0.123	0.341

duced a greater increase in PAH uptake and oxygen consumption in the presence of acetate than in the absence of this substrate.

Since the more active growth hormone preparations contained some TSH it was essential to determine whether the presence of TSH could explain the effects of growth hormone. Thyroidectomized-hypophysectomized rats were given growth hormone (Armour, Lot R 377237) and the results are given in Figure 2. It is clear that thyroidectomy reduced the response to this growth hormone preparation but did not eliminate it completely, the effects of TSH were completely eliminated. This experiment suggested that the TSH content of growth hormone although contributing to the restoration of PAH transport was not the only responsible factor and either growth hormone *per se* or some as yet unidentified renotrophic factor in growth hormone was responsible for some of the restoration of the function under study.

The body weight response to growth hormone administration of thyroidectomized-hypophysectomized rats was 11 grams while that of hypophysectomized rats was 23 grams. Thus thyroidectomy also interfered with

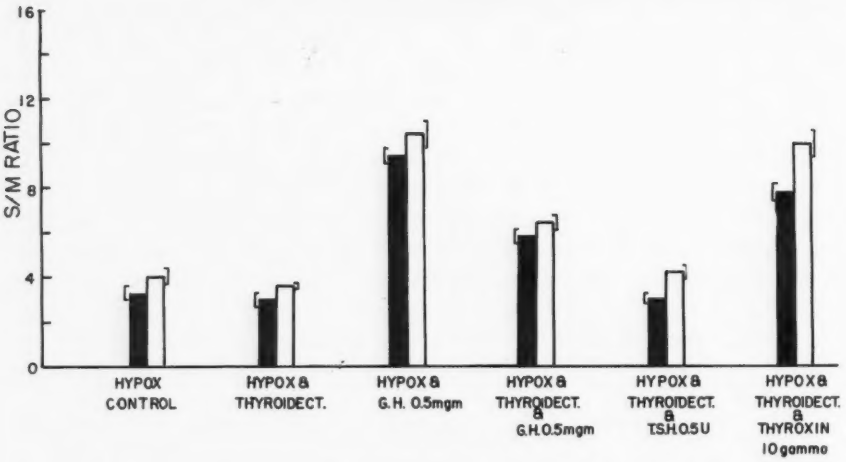

FIG. 2. The effect of thyroidectomy on the renal response of male hypophysectomized rats. PAH S:M ratio was measured and for each group 6–8 rats were used. Dark column: No added substrate. Light column: 0.01 *M* sodium acetate. Growth hormone (GH) used was Armour, Lot R 377237; 0.5 mg. given twice daily for 5 days. Thyroid stimulating hormone (TSH) used was Armour, Lot 491030; 0.5 units given twice daily for 5 days. Thyroxin, 10 gamma once daily for 5 days.

the body weight response to growth hormone and this finding confirms the data of Scow, *et al.* (1949).

From Table 2 it is apparent that GH preparations containing no detectable TSH were less effective in restoring PAH uptake than those prepara-

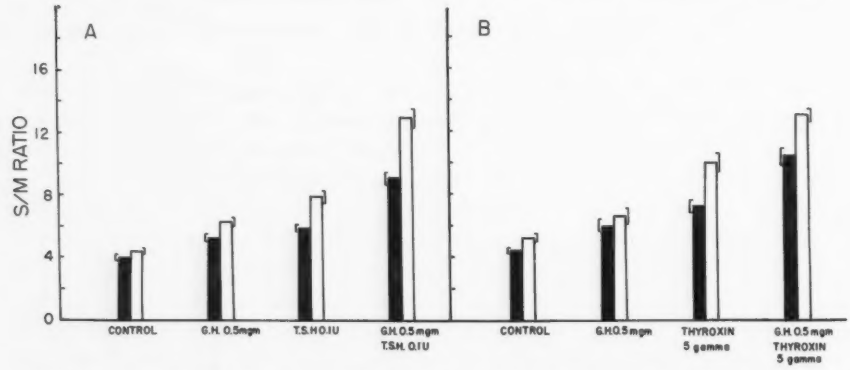

FIG. 3. The effect of combined therapy on the PAH S:M ratio of renal slices from hypophysectomized male rats (29–36 days post-hypophysectomy). A. The effects of growth hormone (GH) and thyroid stimulating hormone (TSH). GH Armour, Lot 369-202B 0.5 mg. and TSH (Armour, Lot 491030) 0.1 units twice daily for 5 days. B. The effects of growth hormone and thyroxin. GH Armour, Lot 369-202B 0.5 mg. twice daily and thyroxin 5 gamma daily for 5 days.

tions containing TSH. The results in Figure 3 show that combining GH with TSH or thyroxin increased the effects on renal PAH uptake and produced a restoration to normal levels.

The luteinizing hormone when given to hypophysectomized male rats increased PAH uptake (Table 5). This observation suggested that testicular function may have some effect on renal PAH transport. We have thus studied the effects of orchidectomy and substitution therapy on PAH transport. Orchidectomy reduced PAH transport significantly especially in the absence of added substrate and this defect could be corrected by the administration of testosterone (Table 8). The administration of

TABLE 8. THE INFLUENCE OF ORCHIDECTOMY, HYPOPHYSECTOMY AND SUBSTITUTION THERAPY ON THE RENAL TRANSPORT OF PAH. MALE SPRAGUE DAWLEY RATS. EXPERIMENTS WERE PERFORMED 25–28 DAYS AFTER ORCHIDECTOMY AND 28–37 DAYS AFTER HYPOPHYSECTOMY

	No. of rats	Body weight in grams	PAH S:M ratio	
			No substrate added	0.01 *M* NaAc
Control	9	183–210	10.3±1.3	14.9±1.6
Orchidectomy 1st exp.	8	310–370	5.8±0.6	10.1±1.4
Orchidectomy 2nd exp.	10	159–228	7.6±0.6	10.1±0.9
Orchidectomy 3rd exp.	18	130–198	6.5±0.8	9.4±0.7
Orchidectomy+testosterone 1 mg. 2× daily for 5 days	12	144–173	9.9±1.1	14.3±0.8
Hypophysectomized	15	86–109	5.2±0.4	6.4±0.5
Hypophysectomized+orchidectomy	6	96–105	5.0±0.6	6.6±0.8
Hypophysectomized+growth hormone Armour, Lot 369202B	6	86– 95	7.0±0.7	9.2±0.6
Hypophysectomized, orchidectomized +growth hormone Armour, Lot 369202B	6	97–107	7.2±0.6	10.0±0.8
Hypophysectomized+testosterone 1 mg. 2× daily for 5 days	8	101–127	7.4±0.3	8.5±0.5

testosterone to hypophysectomized male rats also produced an increase in PAH uptake but did not restore activity to normal levels. In this respect testosterone was qualitatively and quantitatively similar to the pituitary luteinizing hormone.

The possibility that the effects of the purer growth hormone preparations on PAH uptake was due to a contamination with luteinizing hormone had to be considered. No data on the content of LH on the growth hormone preparations was available. We have thus determined the effects of growth hormone Armour, Lot 369202B (contains no detectable TSH), in hypophysectomized-orchidectomized rats and the results are given in Table 8. It is clear that this growth hormone was still active and it is likely that with the growth hormone studied the observed increase in PAH transport was not caused by any LH contaminant.

The observation of Leaf, *et al.* (1952), Earle, *et al.* (1953) and Davis, *et al.* (1954) that ACTH could partially restore PAH clearance in hypophysectomized dogs prompted up to study the effects of adrenalectomy

TABLE 9. THE INFLUENCE OF ADRENALECTOMY, HYPOPHYSECTOMY AND SUBSTITUTION THERAPY ON RENAL PAH TRANSPORT. MALE SPRAGUE DAWLEY RATS WEIGHT 91–127 GM.

	Dosage 2× daily for 5 days	No. of rats	PAH S:M ratio		Post-op. days
			No substrate added	0.01 *M* NaAc	
Control	—	6	10.3±0.6	14.2±0.9	—
Adrenalectomy	—	18	9.5±0.8	14.1±1.1	10–14
Hypophysectomy	—	6	5.0±0.4	6.2±0.6	31–39
Hypophysectomy+Adrenalectomy	—	3	5.2±0.5	6.4±0.3	31–39
Hypophysectomy+GH (Armour, Lot R 377237)	0.5 mg.	4	9.0±0.4	12.8±0.7	31–39
Hypophysectomy, Adrenalectomy+GH (Armour, Lot R 377237)	0.5 mg.	4	9.8±0.3	14.4±0.5	31–39
Hypophysectomy+Cortisone	0.5–1.0 mg.	14	3.4±0.2	4.5±0.4	27–30
Hypophysectomy +DOCA	0.5 mg.	8	4.0±0.3	5.6±0.4	27–30
Hypophysectomy +ACTH Armour Lot 146R-53	1–2 mg.	14	5.3±0.4	6.0±0.4	24–27

and substitution therapy on PAH uptake. The results given in Table 9 show that in the male rat PAH transport was not affected by adrenalectomy nor did ACTH, cortisone or DCA therapy of hypophysectomized rats produce any significant recovery of PAH transport.

DISCUSSION

The results presented show that the depression of PAH transport produced by hypophysectomy was caused by a number of hormonal defects. The data suggest that a depression of thyroid function and testicular function were operative in the production of this defect in PAH transport.

Thyroidectomy produced a reduction of PAH uptake which could be readily corrected to normal levels with thyroxin therapy. TSH or thyroxin administration in hypophysectomized rats produced only a partial restoration of PAH transport. However, combining thyroxin with relatively pure growth hormone produced nearly complete recovery.

The influence of testicular function on PAH transport has been demonstrated. It is thus conceivable that any LH contamination of growth hormone may contribute to the restorative effects of these preparations. These observations are in line with those of Kochakian (1951) and others (see H. W. Smith, 1951) on the action of testosterone on renal weight and enzyme activity.

Whether growth hormone *per se* produceed part of this recovery of PAH transport described is not clear. A number of contaminants have been identified in growth hormone and some of these have definite effects on PAH transport. The evidence presented indicates that a renotrophic substance other than TSH or LH must be found in growth hormone. It is not

possible to state whether growth hormone *per se* is this renotrophic factor. It is of interest that Davis, *et al.* (1954) have shown that a growth hormone preparation containing TSH was more effective in resotoring the renal hemodynamics of hypophysectomized-adrenalectomized dogs than was a GH preparation containing no detectable TSH. Thus our own findings confirm and extend those of Davis, *et al.* and it is thus likely that both in the dog and rat the TSH contaminant of GH contributed to the restoration of PAH clearance towards normal levels.

The evidence presented shows that the adrenal defect following hypophysectomy in male rats is not of significance in the depression of PAH transport. This finding is in contrast to those of Leaf *et al.* (1952), Earle, *et al.* (1953), Davis, *et al.* (1954) in the dog. These investigators have shown an increase in PAH clearance following ACTH therapy of hypophysectomized dogs. The difference in results may be due to species differences or ACTH may have influenced the renal hemodynamics without affecting PAH transport.

The increase in PAH transport following growth hormone therapy could be due to an increase in kidney weight. With all growth hormone preparations studied there was actually an increase in the kidney weight: body surface area ratio of hypophysectomized rats. However, this increase in kidney weight could not be correlated with the increase in PAH transport (Table 2). Furthermore, with the relatively brief TSH and thyroxin therapy period no increase in kidney weight could be demonstrated although PAH transport was increased. It is possible that hormonal therapy which increased PAH transport could have specifically increased the length or size of certain renal tubules and this change could not be reflected in a detectable increase in kidney weight. Oliver (1944–45) has shown that compensatory hypertrophy of the kidney is accompanied by a five fold increase in volume of some proximal tubules although the total increase in kidney weight was only two fold. The effects of growth hormone on the volume of individual renal tubules has not been studied. Grafflin (1939) quotes Turley's finding on a kidney of an acromegalic person where a marked increase in length of the pars convoluta of the proximal tubule was demonstrated. The evidence presented suggests that following growth hormone therapy an increase in the volume of the proximal convoluted tubule may be a partial cause for the increase in PAH transport. By means of cytophotometric determinations of protein-bound sulfhydryl, Cafruny, *et al.* (1955) have shown a decrease in cytoplasmic SH groups of the proximal tubule in the hypophysectomized rat. Furthermore, growth hormone as well as thyroxin therapy produced a partial recovery of this protein-bound sulfhydryl. This evidence indicates that biochemical changes in the individual tubular cells may be operative in the reduction and restoration of PAH transport.

Following hypophysectomy, acetate induced stimulation of PAH transport and oxygen consumption of renal slices was reduced. This findings

indicated an interference with acetate metabolism. Thyroidectomy in its early stages produced only a slight reduction in the acetate stimulation of PAH transport but in later stages showed a defect in acetate utilization. The administration of thyroxin or TSH to hypophysectomized rats had a marked effect on the acetate induced stimulation of both PAH uptake and oxygen consumption. Growth hormone containing no detectable TSH did not produce this specific stimulation of acetate oxidation and utilization. It is thus likely that thyroxin in some way increased the oxidation as well as the utilization of acetate by renal slices. The improvement in acetate utilization following thyroxin therapy was most apparent when small doses of thyroxin were given. Loomis and Lippman (1948) and Martius (1955) have postulated that thyroxin administration produced a dissociation between oxygen consumption and oxidative phosphorylation. The increase in acetate utilization of kidney slices following thyroxin therapy demonstrated in our studies makes it difficult to explain our results by this theory.

Martius (1955) has shown that the prolonged incubation of liver mitochondria in a sucrose solution results in a decrease of oxidative phosphorylation. If the same incubation is made in the presence of low concentrations of thyroxin (10^{-6} *M* or less) this decrease in oxidative phosphorylation is reduced. These *in vitro* observations are contrary to the usual explanations of thyroxin action and it may well be that effects of thyroxin at low concentrations are biologically more significant than those observations obtained with huge dosages of thyroxin. The above mentioned *in vitro* observations of Martius could conceivably be related to the effects of small doses of thyroxin on the acetate induced increase in PAH uptake and oxygen consumption described in this paper.

SUMMARY

Hypophysectomy reduced PAH uptake of renal slices of the rat. The evidence presented indicates that besides a renotrophic factor of pituitary origin (possibly growth hormone) the depression of thyroid and testicular function are operative in the decrease in the PAH transport capacity of the kidney. Adrenalectomy of normal rats had no influence on renal PAH transport. ACTH, cortisone or DCA therapy of hypophysectomized rats did not restore renal PAH transport.

Acetate oxidation and utilization for PAH transport are depressed by hypophysectomy. It is likely that the depression of thyroid function is important in this phenomenon. Small amounts of thyroxin (1–2 micrograms per rat) when given to hypophysectomized rats mainly restore acetate induced stimulation of PAH transport and oxygen consumption.

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SURVIVAL OF ADRENALECTOMIZED MICE AFTER SEVERAL TYPES OF STRESS AND THE EFFECTS OF VARIOUS STEROIDS

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SAUNDERS (1951) reported that lipo-adrenal extract decreased the number of fatalities in sensitized adrenalectomized mice challenged with the antigen while desoxycorticosterone (DOC) offered no protection. These experiments have now been extended to other types of shock using cystalline steroids. Eight means of inducing shock were selected and the protective effects of several representative compounds have been studied. In this paper only the initial phase of shock is considered, and observations are confined to survival rates during the first few hours after a severe stress.

METHODS AND RESULTS

In an attempt to eliminate as many variables as possible, a single species was studied. Adrenalectomized male mice weighing about 25 gm. were used in most tests. As a measure of shock prevention, either the per cent survival in the stressed animals, or average survival time was used. In all cases animals were checked for survival at one hour intervals.

Anaphylaxis: The protective action of lipo-adrenal extract in preventing anaphylaxis in the adrenalectomized mouse was discussed in a previous paper (Saunders, 1951). The effect of cortisone acetate and of DOC was now studied in these animals. In this series the protective steroids were administered at 4 and 2 hours before the intravenous injection of horse serum. Table 1 shows the effectiveness of cortisone in overcoming this type of shock and the lack of activity with DOC. While epinephrine increased the number of survivors, this effect was not statistically significant with the numbers used. However 100% survival was obtained when epinephrine was combined with cortisone acetate, even at the lowest dose of the latter. Dury (1951) suggested that in the shock resulting from an overdosage of glucose, the protective effect of epinephrine was due to a lowering of the blood potassium level. Blood potassium levels were not studied in our series.

Histamine Shock: In an earlier paper, Saunders (1951) showed that the administration of 2 mg. of histamine diphosphate per mouse caused shock in the adrenalectomized, though not in the normal, mouse. This shock could be prevented by various antihistamines. The effect of steroids on this type of shock was further investigated. In this small series the survival rate in the control groups was much higher than that reported in the previous paper. However, cortisone acetate in doses of 0.05 to 0.1 mg. or more, administered at 24 hours and repeated at 4 hours before the histamine, offered some protection against histamine shock. The administration of even 0.4 mg. of DOC under the same conditions was ineffective. Likewise, neither testosterone propionate nor estrone in doses of 0.2 mg. afforded any protection.

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TABLE 1. SURVIVAL OF SENSITIZED ADRENALECTOMIZED MICE AFTER INTRAVENOUS INJECTION OF HORSE SERUM. PROTECTION AFFORDED BY SEVERAL COMPOUNDS

Compound	Dose,* mg.	No. of mice	Per cent survival	χ^{2**}
None	—	95	16	
Cortisone acetate	0.050	21	76	22.47
	0.025	38	58	20.50
	0.01	37	38	6.67
Epinephrine	0.02	9	55	2.69
Cortisone acetate + Epinephrine	0.05 + 0.02	11	100	13.84
Cortisone acetate + Epinephrine	0.025 + 0.02	9	100	11.99
Cortisone acetate + Epinephrine	0.01 + 0.02	10	100	12.99
DOC	0.25	7	22	0.74
DCA	0.05	18	33	0.71

* In all cases this dose of steroid was administered 2 and 4 hours before the horse serum. Epinephrine was administered 15 min. before horse serum.

** Chi-squared was determined by comparison of numbers of surviving animals in the treated groups compared with controls run at the same time. Values ≥ 6.64 indicate significance at 1% level.

Protein Shock: Chen and Wickel (1952) described the production of shock in adrenalectomized rats by the intraperitoneal administration of fresh egg white. They reported that cortisone and hydrocortisone protected rats against this shock, whereas DOC was ineffective, as were also antihistamines and epinephrine. In our experiments, the administration of 0.5 ml. of a 1:1 dilution of fresh egg white to adrenalectomized mice was not followed by shock. All of the control animals survived, as did most of the animals in the treated groups. The experiment was repeated using undiluted egg white and still there were no deaths in the control groups. Apparently the mouse is much more resistant to this type of shock than is the rat.

Glucose Shock: Dury (1951) showed that 2 ml. of a 50% solution of glucose administered by stomach tube to adrenalectomized rats would cause death. Chemical analyses of the blood showed that this was the result of hyperkalemia. Epinephrine afforded protection, while lipo-adrenal extract, in the dosage used, did not. In the present experiment, adrenalectomized mice were treated with a dose of 1 ml. of 50% glucose. The results of several experiments are shown in Figure 1. In ten groups of control animals the average survival rate was $25 \pm 3.7\%$. By calculating values for χ^2, using groups of controls run at the same time as the experimental animals, the protection obtained with 0.02 mg. cortisone acetate and all higher doses was highly significant ($P < 0.01$). However, because of the smaller number of animals with DCA, only the 0.5 mg. dose caused statistically significant protection when compared with the pooled controls. Likewise only the 0.05 mg. dose of hydrocortisone produced significant protection. Thus, while DCA afforded some protection against this type of shock, it was less than 10% as effective as cortisone acetate or hydrocortisone.

Potassium Intoxication: Truszkowski and Duszynska (1940) reported that 0.3 ml. of 2.5% KCl injected intraperitoneally into intact mice weighing 7 to 9 gm. was followed promptly by death. DCA administered 24 and 4 hours before the KCl resulted in an increased number of survivors. In our series, using intact 25 gm. mice, there were no

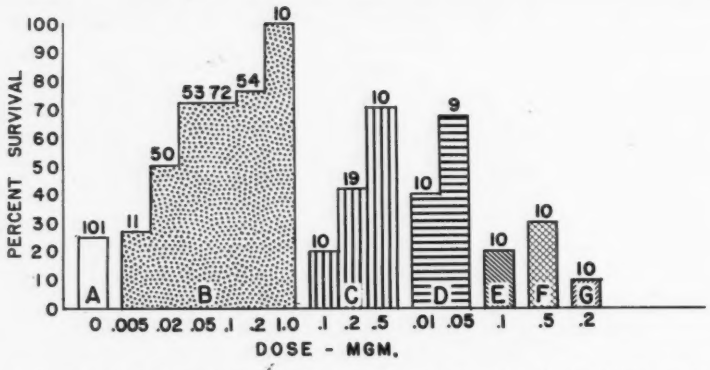

FIG. 1. Survival of adrenalectomized mice, 4 hours after the administration of 1 ml. of 50% glucose by stomach tube. Treatment was instituted as follows: A, oil treated controls; B, cortisone acetate; C, desoxycorticosterone acetate; D, hydrocortisone; E, corticosterone; F, testosterone propionate; G, estrone. The dose was injected 24 hours and repeated 4 hours before the glucose was administered. The numbers above the bars indicate the number of animals at each dose.

survivors after administering 1 ml. of 3.3% KCl and treatment with DCA or cortisone acetate in doses up to 0.2 mg., 2 hours before the KCl, was without effect. With adrenalectomized mice, doses of only 0.5 ml. of 2.5% KCl, i.p., killed all of a group of ten mice within one half hour, while with a dose of 0.1 ml., all of a similar group survived for more

TABLE 2. SURVIVAL OF ADRENALECTOMIZED MICE AFTER ADMINISTRATION OF ½ ML., 2½% KCl, I.P. AND PROTECTION AFFORDED BY VARIOUS COMPOUNDS

Compound	Dose, mg.	Time administered	No. treated mice	Per cent survival	χ^{2**}
None	—	—	101	15*	
Cortisone acetate	0.02	−24 and −4 hrs.	20	45	0.43
	0.025		34	26	3.50
	0.05		52	50	13.93
	0.1		52	50	7.85
	0.2		51	65	26.99
Desoxycorticosterone	0.02	−24 and −4 hrs.	19	37	0.01
	0.025		33	24	2.78
	0.05		40	55	12.95
	0.1		49	53	9.32
	0.2		53	66	27.02
Testosterone propionate	0.2	−24 and −4 hrs.	32	19	0.96
Estrone	0.2	−24 and −4 hrs.	31	23	0.13
Epinephrine	0.005	−5 min.	16	50	0.77
	0.01		40	45	2.44

* In no group of 10 control mice was the survival rate over 30%.

** Chi-squared was determined from the numbers of animals surviving in the treated groups and in control groups run at the same time. Values $\geqq 6.64$ indicate significance at the 1% level.

than four hours. In another series of adrenalectomized mice, the survival rates after various doses of KCl administered i.p. were: 500 mpk, 48%; 600 mpk, 30%; 700 mpk, 14%. The mice in this series were somewhat larger, and this may account for the slightly higher survival rate. For the succeeding experiments, a dose of 0.5 ml. of 2.5% KCl per mouse was used to induce shock. To study the protective effect of steroids, the dose was divided and administered in oil, 24 and 4 hours before KCl. Both DOC and cortisone acetate afforded protection (Table 2), but estrone and testosterone propionate did not. It may well be that DOC, by its influence on mineral metabolism, promotes a potassium diuresis or a redistribution of potassium, thus overcoming the toxic effects of this ion.

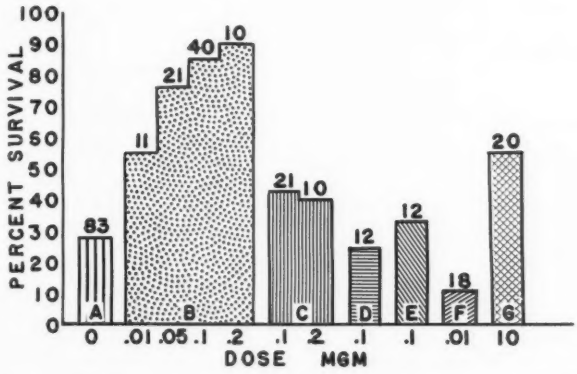

FIG. 2. Survival of adrenalectomized mice, 4 hours after administration of 0.1 unit of protamine zinc insulin. Treatment was instituted as follows: A, oil treated controls; B, cortisone acetate; C, desoxycorticosterone acetate; D, estrone; E, testosterone propionate; F, epinephrine; G, glucose. The dose indicated was administered 24 and 4 hours before insulin in the case of the steroids. Epinephrine was administered 15 min. and glucose, 30 min. before insulin. The numbers above the bars represent the number of animals at that dose.

On the other hand, cortisone perhaps acts only to restore to normal the resistance of the adrenalectomized animal. As in the series involving anaphylactic shock, here again partial, but not statistically significant, protection was observed after epinephrine treatment. Since the mice were adrenalectomized, it is improbable that the epinephrine acted through the release of ACTH from the pituitary, although extra-adrenal activity of ACTH has been reported (Jacot & Selye, 1952).

Insulin Shock: Hypoglycemic shock was produced in adrenalectomized mice by the administration of 0.1 unit of protamine zinc insulin. The animals were fed *ad libitum* until the insulin was injected, but after this time they were deprived of both food and water. As determined from the calculation of Chi squared, pretreatment with cortisone acetate at doses of 0.05 mg. and above afforded marked protection while the other steroids did not. A single dose of glucose, administered before the insulin, also increased the survival rate (Fig. 2), but this increase was not statistically significant.

Cold Shock: As early as 1931, Hartman *et al.* demonstrated the effectiveness of adrenal steroids in prolonging the survival of adrenalectomized rats exposed to low temperatures. For our experiments on cold shock each mouse was housed in an individual cage, 3″×3″, in a cold room at 3° to 6° C. The animals did not receive either food or water during the test. In the initial experiments it was found that while injection of steroids in oil at the

TABLE 3. AVERAGE SURVIVAL TIME OF ADRENALECTOMIZED MICE EXPOSED TO COLD

Protective compound	Dose,* mg.	No. mice	Survival** time
			hrs. ±S.E.
None	—	108	3.89 ±0.19
Cortisone acetate	0.005	11	4.09 ±0.48
	0.01	75	4.60 ±0.31
	0.02	32	5.59 ±0.42
	0.025	11	5.63 ±0.74
	0.05	85	5.34 ±0.27
	0.1	66	5.35 ±0.31
	0.2	55	7.29 ±0.20
Desoxycorticosterone	0.2	21	3.29 ±0.35
Estrone	0.2	10	3.50 ±0.53
Testosterone propionate	0.2	22	2.36 ±0.29
Corticosterone	0.05	11	4.00 ±0.47

* This dose was administered at −24 hrs. and repeated at 0 hrs.
** The summation of the no. of mice surviving at the end of each hour up to 8 hrs. divided by the initial number of mice.

beginning of the exposure to cold had no protective effects, the same total dose divided and administered 24 and 0 hours before exposure was effective. The results presented in Table 3 were obtained with adrenalectomized mice, treated with the steroid on the preceding day as well as immediately before exposure to cold. Under these conditions cortisone acetate showed definite protection at doses as low as 0.01 mg., and the survival time was increased at higher dosage. None of the other steroids afforded protection. Figure 3 illustrates a typical experiment in which the effect of increasing doses of cortisone is well demonstrated.

Snake Venom Poisoning: In a preliminary series it was found that doses of 0.01 to 5 μg. of dried snake venom (Crotalus viridis) per mouse produced no fatalities within a period of 6 hours after intraperitoneal injection into adrenalectomized mice. Doses of 10 to 50 μg. produced increasing mortality. We therefore selected a dose of 50 μg. for pro-

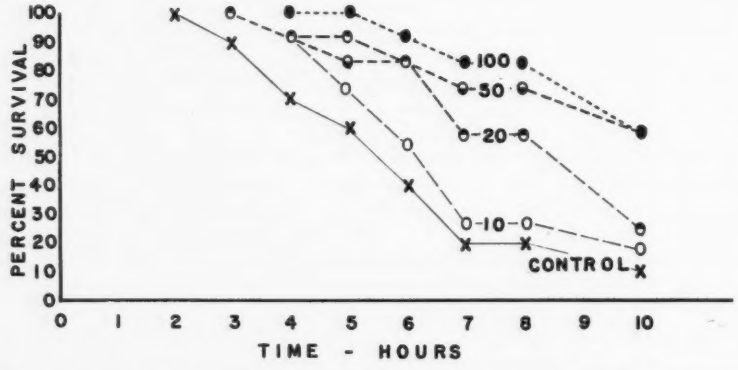

FIG. 3. Effect of cortisone acetate on the survival of adrenalectomized mice exposed to cold. The figures indicate the dose in micrograms, administered to each mouse at 24 and 0 hours before exposure to cold.

TABLE 4. SURVIVAL OF ADRENALECTOMIZED MICE AFTER TREATMENT WITH SNAKE VENOM

Protective compound	Dose,* mg.	No. mice	Per cent survival
None	—	10	10
Cortisone acetate	0.025	10	40
	0.1	10	70**
DOC	0.1	10	30
Epinephrine	0.01	10	10
Cortisone acetate + Epinephrine	0.025 + 0.01	10	20
Diphenhydramine	0.5	10	10
Glucose	100	10	0

* Steroids were administered at 24 and 2 hours before, other compounds 15 min. before, snake venom.

** Significant at the 5% level compared to controls by the Chi-squared method. No other values are significantly different from the controls.

TABLE 5. SUMMARY OF THE ACTIVITY OF VARIOUS COMPOUNDS AGAINST DIFFERENT FORMS OF SHOCK IN ADRENALECTOMIZED MICE

Protective compound	Anaphylaxis	Histamine	Glucose	KCl	Insulin	Cold	Snake venom
Cortisone acetate	+	+	+	+	+	+	+
6-dehydrocortisone acetate						+	
Hydrocortisone			+				
11β,17α-dihydroxyprogesterone	+		+				
9-chloro-11β,17α-dihyroxyprogesterone			+	−		+	
Corticosterone	+		−			±	
11-desoxycorticosterone	−	−	+	+	±	−	±
11-desoxycorticosterone acetate	±		+				
19-nor,11-desoxycorticosterone acetate		−		−	−	±	
17aα-ethyl-17a-hydroxy-D-homo-4-androsten-3-one						−	
16β-formyloxy-17-hydroxyprogesterone			+				
3-methoxy-17-(β-hydroxyethylidene)-1,3,5(10)-estratriene				−		±	
4-androstene-3,17-dione	−						
11-hydroxy-4-androstene-3,17-dione	−						
Testosterone propionate			−	−	−	−	
Testololactone	−						
Estrone			−	−	−	−	
4′-benzyloxy-α,α-diethyl-4-stilbenol	−						
2,3-bis(p-hydroxyphenyl)valeronitrile	−					−	
3,4-bis(p-hydroxyphenyl)2-hexanone	−					±	
N,N-diethyl-2,6-leupetidinium bromide	−						
Propantheline bromide	−						
Diphenhydramine	+	+					−
Glucose					+		−
Epinephrine	+		−	+	−		−

Doses and time of administration were variable.
+ = Definite increase in per cent surviving.
± = Questionable effect.
− = No increase in survival rate.

duction of shock. Cortisone produced definite protection and DOC also increased the per cent of survivors (Table 4). That histamine production was not responsible for this shock is indicated by the ineffectiveness of the antihistamine, diphenhydramine, in its prevention.

Miscellaneous Compounds: Table 5 summarizes the protective effects of a wide variety of compounds tested in one or more of the types of shock previously discussed. Various doses were used so the activities reported are not strictly comparable. It will be noted that protection was afforded by the antihistamine, diphenhydramine, against anaphylactic and histamine shock, glucose against insulin shock, epinephrine against KCl and anaphylactic shock, DOC against glucose and KCl shock, and 16-β-formyloxy-17-hydroxyprogesterone against glucose shock. Of the other compounds tested, only the 11-oxygenated steroids afforded definite protection.

DISCUSSION

Halpern *et al.* (1952) have reported that DOC does not modify the high sensitivity of adrenalectomized mice to histamine whereas cortisone and epinephrine increase this tolerance. These authors also reviewed other studies on the effects of adrenal hormones in several types of shock.

The types of stresses used in our study may be grouped as follows: first, those that induce hypoglycemia. In this group are included insulin shock and cold shock. In both of these cases cortisone is very effective, whereas DOC seems to be without effect. In the second group are those means of producing shock which appear to involve an increase in the potassium of the blood. This may be induced by injection of potassium chloride or by the administration of excess glucose, as shown by Dury. In both of these cases cortisone is very active, but DOC is also effective. The third group involves those methods which appear to involve histamine in the reaction. In this group are included histamine shock, anaphylaxis, the administration of snake venom or the administration of egg white. In all of these cases cortisone is very effective, while DOC is without effect, with the possible exception of the experiments with snake venom. From the lack of the effectiveness of diphenhydramine in this latter experiment, it appears doubtful that snake venom works through a histamine mechanism.

In all of these experiments adrenalectomized mice were used. In all types of shock, the adrenalectomized mouse is more sensitive than is the normal mouse. Treatment with cortisone restores the resistance of the adrenalectomized mouse toward normal, while DOC is effective only when the mineral balance of these animals has been markedly upset. Because of the high resistance of normal mice to all of these types of stress, this work was not repeated using normal animals.

SUMMARY

Adrenalectomized mice were subjected to various types of stress. The protective action of cortisone acetate and desoxycorticosterone were studied in all cases. In some experiments the effect of estrone, testosterone propionate and epinephrine were also followed. Stress which involves hyper-

kalemia was induced either by injection of KCl or by administering large doses of glucose by stomach tube. In both cases cortisone acetate and desoxycorticosterone are equally effective. In stress involving hypoglycemia, insulin administration or exposure to cold, cortisone acetate is effective, while desoxycorticosterone is not. Cortisone acetate also affords protection against histamine shock, anaphylactic shock and snake venom poisoning, whereas desoxycorticosterone does not.

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BIOASSAY OF THYROTROPIN[1]

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MOST of the procedures for the biosassy of thyrotropin involve subcutaneous or intraperitoneal administration of a series of test doses of the hormone over a period of 3 to 5 days (*cf.*, Albert, 1949; Turner, 1950). Ghosh *et al.* (1951) developed an assay based on the uptake of I^{131} by the thyroid of the hypophysectomized rat following a series of 5 subcutaneous injections of thyrotropin. Subsequently, Overbeek *et al.* (1953) substituted rats prefed with iodocasein for hypophysectomized animals, and administered thyrotropin in 2 intraperitoneal doses. A ten-fold increase in sensitivity was achieved over the method of Ghosh *et al.* (1951). Recently, Kriss and Greenspan (1954) described a bioassay based on the enhanced uptake of P^{32} by the chick thyroid following a single intracardiac injection of thyrotropin. The sensitivity of this method was about 10 times that observed after a single intraperitoneal injection of thyrotropin (Crooke and Matthews, 1953).

The present studies were initiated by our desire to have a method for the bioassay of thyrotropin which would satisfy the following criteria:

1. Maximum sensitivity and precision to permit measurement of minute quantities of thyrotropin in biological material.
2. Linear response over an extended range of dosage in order to facilitate dilution of unknown samples into the effective range.
3. Response to a single injection of thyrotropin, thus eliminating the need for prolonged storage of samples of uncertain stability.

The bioassay procedures reported below were examined for their ability to fulfill these requirements, and are of a preliminary nature. Measurements have been made of I^{131} uptake by rat thyroid glands after a single intravenous injection of thyrotropin, utilizing 4 preparations: (1) the intact, thyroxine-treated rat, (2) the intact thyroxine plus propylthiouracil-treated rat, (3) the hypophysectomized rat, and (4) the intact rat prefed with thyroid power. A preliminary report of this study has been presented (Levey *et al.*, 1955).

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MATERIALS AND METHODS

Male Sprague-Dawley rats weighing 100–150 gm. were used. Intact rats were maintained on Purina Laboratory Chow. Hypophysectomized rats (purchased from the Hormone Assay Laboratories, Chicago, Illinois) were fed ground horse meat, milk, bread, and apples. No attempt was made to control iodine intake. DL-thyroxine (25 μg/rat/day) was administered subcutaneously in alkaline saline solution; thyroid powder was administered in ground Purina Chow; propylthiouracil (5 mg/rat/day) was given subcutaneously in saline suspension containing Tween 80 as an emulsifying agent; carrier-free I^{131} was administered intraperitoneally in saline solution; and thyrotropin (Armour's Thytropar) was injected intravenously by the saphenous or tail vein route in saline solution. In one experiment (*cf.*, Fig. 2), thyrotropin was administered subcutaneously in saline solution. A volume of 0.2 ml. was employed for all injections.

At sacrifice, the thyroid glands were removed, weighed, dried on aluminum planchets, and counted with a shielded Geiger tube. In some cases, Geiger counting was carried out on aliquots of an alkaline digest of the thyroid tissue, or scintillation counting was performed on dried, intact thyroid glands. Neither of the latter two methods increased the overall accuracy of the assay, although the alkaline digests yielded higher total counts for each thyroid. All the data reported below are based on Geiger counting of the dried, intact glands.

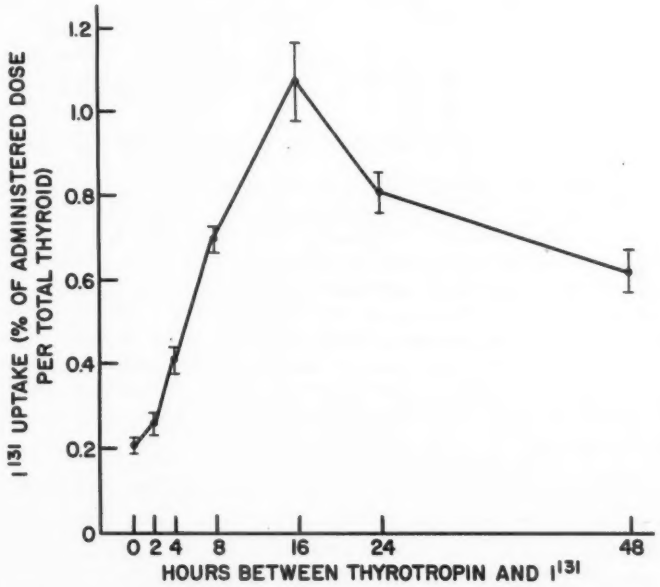

FIG. 1. Time study on the effect of a single intravenous injection of thyrotropin on the 4-hour uptake of I^{131} by the thyroid of the thyroxine-treated rat. Animals were pretreated with 25 μg. of DL-thyroxine per day for 6 days, followed by an intravenous injection of 50 milliunits (U.S.P.) of thyrotropin on the 6th day. Two μc. of I^{131} were administered on the morning of the 7th day. Each group contained 6 animals. Standard error of the mean is shown for each group.

RESULTS

The pituitary gland of the rat is relatively high in thyrotropic hormone activity (Adams, 1946). In order to utilize this animal for the bioassay of thyrotropin, some means must be employed to reduce endogenous thyroidal activation by the pituitary. In the present studies, this aim was

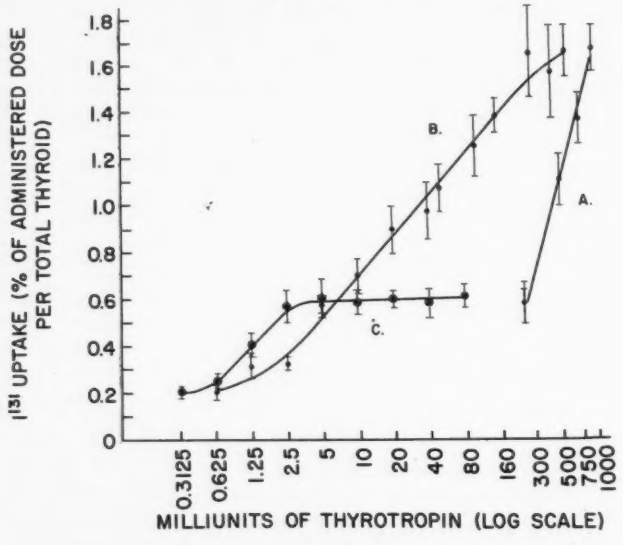

FIG. 2. Dose-response curves. A. Rats were pre-treated with DL-thyroxine (25 μg./day) for 7 days. Thyrotropin was administered subcutaneously in 5 daily divided doses concurrent with the last 5 injections of thyroxine. Four-hour I^{131} uptakes were determined on the day following the last injection. Each group contained 8 animals. B. Rats were pre-treated with DL-thyroxine (25 μg./day) for 7 days. Thyrotropin was administered in a single intravenous injection on the 7th day. Four-hour I^{131} uptakes were determined 20 hours after the thyrotropin injection. Each group contained 7 animals. C. Rats were hypophysectomized 2 weeks prior to use. Four-hour I^{131} uptakes were determined 20 hours after administration of a single intravenous dose of thyrotropin. Each group contained 5 animals. Two μc. of I^{131} were given in all cases. Standard error of the mean is shown for each group.

achieved by hypophysectomy, thyroxine preinjection of the intact rat, or thyroid powder pre-feeding of the intact rat. Fig. 1 illustrates the effect of a single intravenous injection of 50 milliunits (U.S.P.) of thyrotropin on the 4-hour uptake of I^{131} by the thyroid glands of the thyroxine-treated rat. Maximal stimulation of uptake was seen 16 hours after thyrotropin administration. The advisability of employing the 4-hour period for I^{131} uptake is indicated by Fig. 1. With maximal stimulation of I^{131} uptake occurring about 16 hours after the administration of thyrotropin, prolonging the period between injection of the isotope and sacrifice would result in I^{131} uptake measurements being made at a time when the thyro-

tropic effect had long passed its peak. Fig. 2 demonstrates that subcutaneous administration of thyrotropin in divided doses (curve A) is 10–15 times less effective than a single intravenous dose (curve B) in stimulating uptake of I^{131} by the thyroid glands of thyroxine-treated rats. However, the subcutaneous route of administration resulted in a steeper response curve with good precision ($\lambda = 0.160$). The hypophysectomized

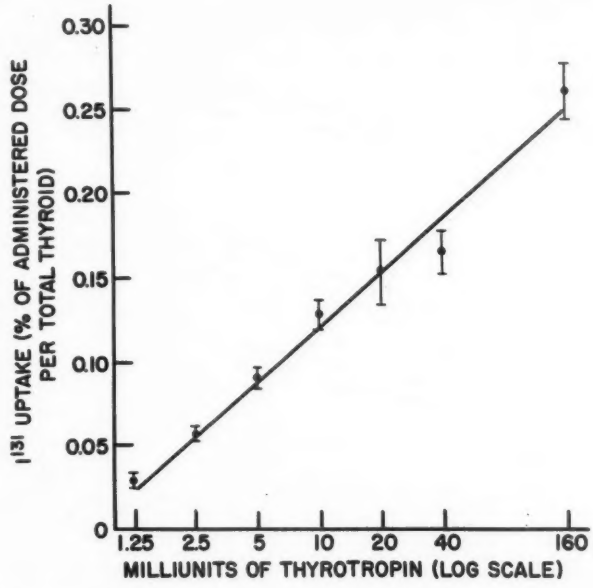

FIG. 3. Dose-response curve. Animals were pretreated with DL-thyroxine (25 μg./day) and propylthiouracil (5 mg./day) for 5 days. Thyrotropin was administered intravenously on the 5th day. Propylthiouracil was given on the 6th day followed by 5 μc. of I^{131}. The rats were sacrificed 2 hours later. Each group contained 6 animals. Standard error of the mean is shown for each group.

animal showed the most sensitive response to intravenous thyrotropin (curve C). The effective range of 0.625–2.5 milliunits ($\lambda = 0.240$) was comparable to that reported for P^{32} uptake by the chick thyroid following intracardiac injection of thyrotropin (Kriss and Greenspan, 1954).

The possibility existed that isolation of the iodide-trapping mechanism of the thyroid by blocking the formation of organic iodine with an antithyroid agent such as propylthiouracil (*cf.*, Astwood, 1944–45; McGinty, 1949) might result in enhanced sensitivity and accuracy of the response to intravenously-administered thyrotropin. Accordingly, rats were treated with thyroxine and propylthiouracil as described in the legend to Fig. 3. The 2-hour time interval between injection of I^{131} and sacrifice was selected in view of previous reports (VanderLaan and Bissell, 1946; Chaikoff and

Taurog, 1949) that iodide uptake by the propylthiouracil-blocked thyroid was maximal by this time. This assay was sensitive to 2.5 milliunits of thyrotropin and showed satisfactory precision ($\lambda = 0.229$). A study was conducted on I^{131} uptake following intravenous administration of thyrotropin to animals prefed with thyroid powder. As seen in Fig. 4, 0.005% thyroid powder did not suppress thyroid activity, and 0.2% was too inhibi-

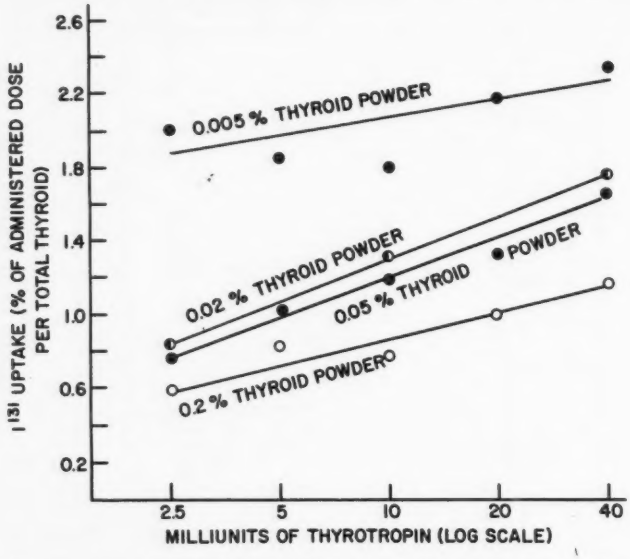

FIG. 4. Dose-response relationships in thyroid powder pre-fed rats. Thyroid powder pre-feeding was carried out for 10 days, followed by a single intravenous injection of thyrotropin. Two μc. of I^{131} were administered 20 hours later. The rats were sacrificed 4 hours after receiving the isotope. The mean value is shown for each group (6 rats per group).

tory. From these and additional observations, 0.02% appeared to be borderline, while 0.05% was consistently inhibitory without masking the stimulating effect of exogenous thyrotropin. The curve in Fig. 5 is a composite of 3 runs using animals prefed with 0.05% thyroid powder. The ordinate is expressed as stimulated/control I^{131} uptake in order to minimize differences in the control levels.

DISCUSSION

In the present studies, the enhanced sensitivity of the thyroidal response to thyrotropin administered by the intravenous as opposed to the subcutaneous route is in accord with the report of Sayers *et al.* (1948) on the advantages of the intravenous route for the bioassay of adrenocorticotropin. Maximal enhancement of thyroidal I^{131} uptake was seen 16 hours after intravenous administration of thyrotropin as compared with 48 hours

following subcutaneous injection (VanderLaan and Greer, 1950). That the thyroid gland of the thyroxine-treated rat did not respond to exogenous thyrotropin in the same fashion as that of the hypophysectomized rat was indicated by the significantly lower threshold and narrower dose range observed in the latter animal. Although thyroxine pretreatment achieved the desired effect of suppressing endogenous production of thyrotropin, the

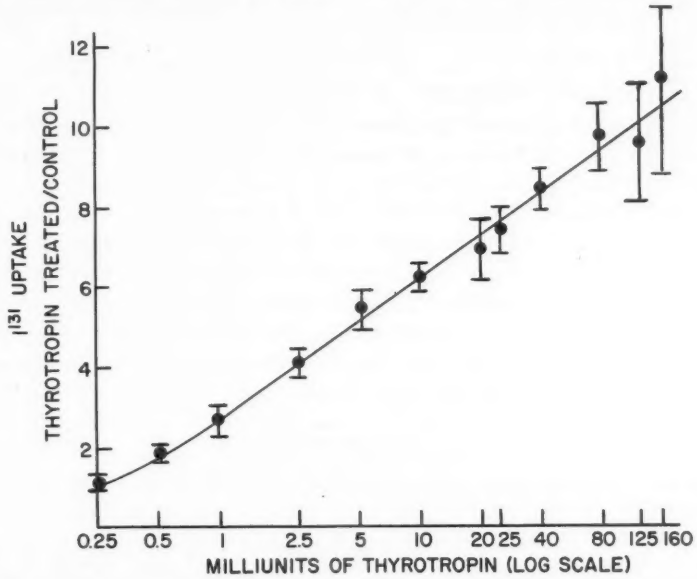

FIG. 5. Dose-response curve. Rats were pre-fed for 10 days with stock diet containing 0.05% thyroid powder. Four-hour I^{131} uptakes were determined 20 hours after administration of a single intravenous dose of thyrotropin. Since the above curve is a composite of 3 runs, the thyrotropin response was expressed as a multiple of the control response. Standard error of the mean is shown for each group (6–8 rats per group).

higher threshold of the thyroidal response to exogenous thyrotropin suggests modification of the iodine trapping and/or binding mechanism. This modification might be attributed either to the flood of exogenous thyroxine or to pituitary factors other than thyrotropin which exert an influence on the thyroid. For example, there is evidence (Woodbury *et al.*, 1951; Rawson, 1952) that, in the hypophysectomized rat, adrenocorticotropin can inhibit the response of the thyroid to exogenous thyrotropin; and there are conflicting reports that thyroxine may (Cortell and Rawson, 1944) or may not (Halmi *et al.*, 1953) lower the thyroid iodide-trapping capacity of hypophysectomized rats treated with thyrotropin. However, more recent evidence (Halmi and Spirtos, 1954) implies that an intrinsic thyroid principle, possibly the thyroid hormone itself, may inhibit the iodide-trapping mechanism.

In general, we favor the thyroid power prefed rat as an assay animal because of the convenience of preparation, the pronounced response to a single intravenous injection of 0.5 milliunits of thyrotropin, and the linear response over an extended range of dosage. We have utilized the method extensively for the bioassay of pituitary extracts and thyrotropin-fortified serum samples, but have encountered difficulty with inhibitory substances in materials of biological origin which tend to flatten the response curve.

SUMMARY

Studies have been conducted on the uptake of I^{131} by the rat thyroid following a single intravenous injection of thyrotropin. Dose-response relationships were determined utilizing thyroxine-treated, thyroxine plus propylthiouracil-treated, thyroid powder prefed, and hypophysectomized rats as assay animals. The thyroxine-treated rat was least sensitive of all. The hypophysectomized rat responded linearly over the range of 0.625–2.5 milliunits (U.S.P.) of thyrotropin. The thyroxine plus propylthiouracil-treated rat was less sensitive than the hypophysectomized animals, but responded over a greater range of dosage (2.5–160 milliunits). The thyroid powder prefed rat was most convenient to deal with and responded linearly over the range of 0.5–160 milliunits.

Acknowledgment

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EFFECT OF PANTOTHENIC ACID DEFICIENCY ON INSULIN SENSITIVITY AND RESPONSE TO ACTH OF INTACT AND DIABETIC RATS[1]

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INTRODUCTION

DEANE and McKibbin (1946) observed in the adrenal cortex of pantothenic acid deficient rats cytochemical changes which were consistent with an early phase of adrenocortical hyperfunction followed by a phase of exhaustion and adrenocortical hypofunction. These observations have stimulated many investigators to study the functional capacity of the adrenal cortex in this deficiency state. However, only a few studies have been reported which are concerned specifically with the effects of this deficiency state on those phases of carbohydrate metabolism influenced by the adrenal cortex. Irwin and Ralli (1948) were unable to find any effect of pantothenic acid deficiency on the severity of the diabetes of a small group of rats with alloxan diabetes. Hurley and Morgan (1952) demonstrated a decreased ability of the liver to store glycogen and a lowered blood glucose in rats with acute pantothenic acid deficiency subjected to anoxia. Since these abnormalities were corrected by administration of adrenal cortical extract (ACE), Hurley and Morgan interpreted these findings as indicating impairment of adrenal cortical function. Winters, Schultz and Krehl (1952) reported that phloridzinized pantothenic acid deficient rats were incapable of excreting glucose and nitrogen in amounts comparable to non-deficient pairfed controls and that there was an increased sensitivity to insulin in these deficient rats. These findings also could be due to adrenal cortical hypofunction.

It is the purpose of this paper to report the effects of pantothenic acid deficiency on the diabetic status of the partially depancreatized diabetic rat, the results of insulin sensitivity studies on diabetic and non-diabetic rats suffering from mild, moderate and severe degrees of pantothenic acid deficiency, and the effects of administering ACTH to pantothenic acid deficient diabetic rats.

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METHODS

All animals used in these experiments were male rats of the Long-Evans strain which were placed on a purified diet deficient in or supplemented with pantothenic acid.[2]

Rats used for determining the insulin sensitivity of non-diabetic animals were placed on the pantothenic acid deficient or control diet at 26 to 31 days of age. Except for one experiment, in which the controls were fed *ad libitum*, all control animals were pairfed with their experimental pairmates. At the end of 8 to 14 weeks the animals were fasted 24 hours, placed under moderate sodium pentabarbital anesthesia, the lower vena cava exposed and insulin (Lilly) was injected at the level of 0.1 units per kilogram body weight. Blood samples were taken at the time of injection and every 30 minutes thereafter for at least two hours. All animals were kept on a constant temperature (38° C) plate during the time of taking blood samples. Blood glucose was determined by the colorimetric method of Nelson (1944).

The diabetic rats used were operated on at the age of 27 to 32 days and all visible pancreas was teased away with toothpick type swabs. The animals were then replaced on a complete stock diet for 30 days. Animals which at this time were diabetic were paired as to both food consumption and level of glycosuria and then were placed on the deficient or control diets. In three separate studies the deficient groups remained on the diets 14, 19 and 23 weeks respectively.

All animals studied were kept in individual, screen-bottom metabolism cages which were tightly fitted on urine collection funnels. In the studies with diabetic animals urine collections were made every 24 hours at the time of feeding. Total reducing substances were determined by the method of Somogyi (1945) and total urinary nitrogen by the modified micro-Kjeldahl method. When insulin was given, protamine zinc insulin (Lilly) was injected, in amounts indicated on the graphs, one to two hours after feeding for 4 or 5 successive days.

All animals on the deficient diet exhibited typical symptoms of pantothenic acid deficiency before they were used in an experiment. Graying of the fur, "spectacled eyes," dermatitis and prophyrin deposition were used as criteria of the deficiency. Many more animals were placed on the deficient diets than those reported in this study, as all possible efforts were taken to standardize the animals according to degree of deficiency, as judged by macroscopic appearance and food intake.

RESULTS

Insulin sensitivity of pantothenic acid deficient intact rats. The first group of 5 pantothenic acid deficient and 7 *ad libitum* control animals was tested after 8 weeks (54 days) on their respective diets. Figure 1-A shows that the deficient rats were resistant to the effects of the insulin injection. The

[2] The pantothenic acid deficient diet was composed of 24% alcohol-extracted casein, 64% sucrose, 8% hydrogenated vegetable oil (Crisco or Primex), and 4% salts no. 4. Crystalline vitamins per kilogram of diet were: 300 μg. D-biotin, 5 mg. 2-methyl-1, 4-naphthoquinone, 5 mg. thiamine HCl, 5 mg. pyridoxine HCl, 5.5 mg. pteroylglutamic acid, 10 mg. riboflabin, 10 mg. p-aminobenzoic acid, 20 mg. niacin, 400 mg. inositol, and 1.0 gm. choline chloride. The control diet contained the identical constituents but was supplemented with 50 mg. D-calcium pantothenate per kilogram diet. All rats received weekly a fat-soluble vitamin mixture containing 800 I.U. vitamin A, 115 chick units vitamin D, 6 mg. synthetic DL-alpha-tocopherol, and 650 mg. corn oil (Mazola). We are greatly indebted to Dr. Thomas H. Jukes, Lederle Laboratories, to Dr. Randolph Major of Merck and Company, Inc. and to Dr. Elmer L. Sevringhaus of Hoffman LaRoche, Inc. for generous supplies of crystalline vitamins.

deficient animals had an average decrease of only 13 mg.% in blood sugar level as compared with the decrease of 31 mg.% in the *ad libitum* controls. When the data shown in 1-A are compared statistically the p values for the four points after insulin administration are 0.01, 0.03, 0.20 and 0.03 respectively. To determine whether or not this resistance to insulin was

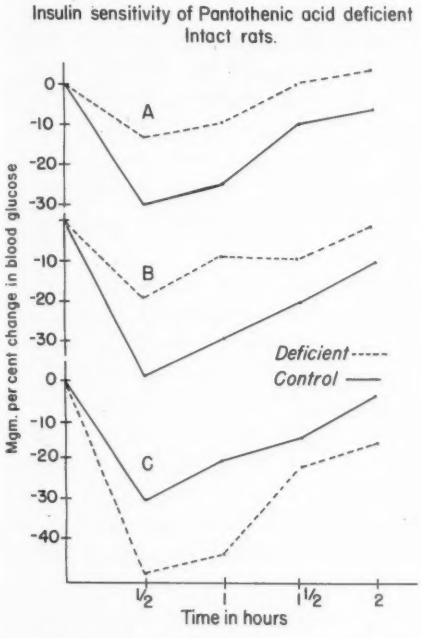

FIG. 1. In this graph are plotted the average changes in blood sugar following the administration of 0.1 units of insulin per Kg of body weight. Each curve is the average of 5–7 animals. A compares animals after 54 days of deficiency with their *ad libitum*-fed controls. B compares animals after 54 days of deficiency with their pairfed controls. C compares animals after 98 days of deficiency with their pairfed controls.

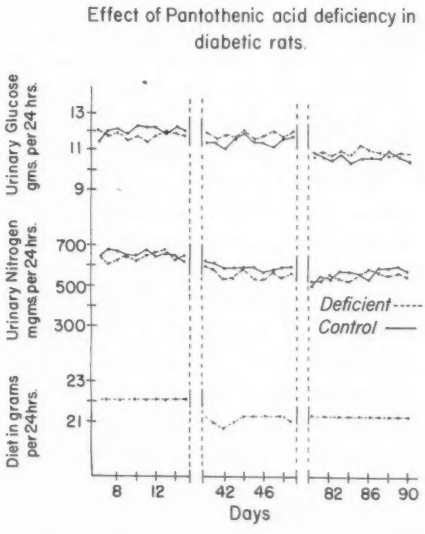

FIG. 2. Each curve represents the average of 4 diabetic rats. The food intake, urinary glucose, and urinary nitrogen over a period of 90 days on the purified diets are shown. The lack of effect of pantothenic acid deficiency *per se* on the severity of the diabetic status as judged by glycosuria and urinary nitrogen is demonstrated.

due to the decreased food consumption accompanying the deficiency, 5 rats were pairfed with the next group of deficient animals and the groups were tested after the same experimental period, namely 8 weeks. The data from this experiment are presented in Figure 1-B and show that the insulin sensitivity of these animals was like that of those in the previous group. The deficient animals showed a decrease in blood glucose of only 19 mg.% whereas the pairfed controls exhibited a decrease of 39 mg.%. Again when

the data were compared statistically p values of less than 0.01 were obtained for the first two points after insulin administration. In addition, the blood sugar of the deficient animals returned to normal sooner than that of the pairfed control rats. The third group of animals, 5 deficient and 5 pairfed control rats, was tested after 14 weeks (98 days) on the experimental diets. In contrast to the two previous deficient groups which had shown a resistance to insulin, this group of animals, deficient for a longer period of time, exhibited an increased sensitivity to insulin with an average decrease in blood sugar of 47 mg.%. The response to insulin of these deficient animals was more variable than that of those in the previous groups. Thus statistical analysis revealed only one point, *i.e.* at $\frac{1}{2}$ hour after insulin, when the difference between the two groups was significant. Here the p value was 0.04. The pairfed controls showed a similar response to insulin as had the controls of the previous groups, namely, a drop of 32 mg.% in blood sugar (Fig. 1-C).

Effect of pantothenic acid deficiency in diabetic rats. Eight diabetic animals were used in this study and all had a definitely established glycosuria before being placed on the deficient and control diets at 60 days of age. Part of the data from this experiment are presented in Figure 2. During 96 days of deficiency there was a slight decrease in food consumption in the deficient animals with a corresponding decrease in excreted nitrogen and glucose. Taking this change into account there was no difference between the deficient and control groups with respect to the severity of the diabetes. Subsequently these animals were given insulin for 4 days and during the post-injection control period of an additional 4 days the severity of their diabetes was still unaltered. Thus, no effect of pantothenic acid deficiency *per se* on the severity of the diabetic state was observed during 104 days of continuous metabolic observation.

Effect of insulin in pantothenic acid deficient diabetic rats. The first group of diabetic animals, 4 deficient and 4 pairfed controls, was injected with protamine zinc insulin after 14 weeks (96 days) on the experimental diets. Each animal received 2 units per day for 4 days. Figure 3 shows that in the pairfed control group the glycosuria decreased from an average preinjection level of 10.4 gm. per rat per day to a low point of 3.4 gm. (67% decrease) during insulin therapy. In the deficient group the glycosuria decreased from an average preinjection level of 11.0 gm. to only 7.6 gm. (31% decrease) during insulin therapy. The difference in average glycosuria for the two groups during insulin therapy was statistically significant, the p value being less than 0.01. Thus, the deficient rats were less sensitive to insulin than those on the control diet. The two groups showed no difference in urinary nitrogen excretion throughout the experiment.

The second group of diabetic animals, 5 deficient and 5 pairfed controls, was injected with 2 units protamine zinc insulin per rat per day for 5 days after 19 weeks (130 days) on the purified diets. Figure 4 shows that in the pairfed control group the glycosuria decreased from an average preinjec-

tion level of 10.3 gm. per rat per day to a low of 4.6 gm. (60% decrease) during insulin therapy. In the deficient animals the glycosuria decreased from the average preinjection level of 12.0 gm. per rat per day to a low of 4.0 gm. (67% decrease) during the injection period. There were no significant differences between the deficient and the control groups in either glucose or total urinary nitrogen excretion during the injection period. Thus, in this group of animals the deficiency did not result in any significant change in insulin sensitivity.

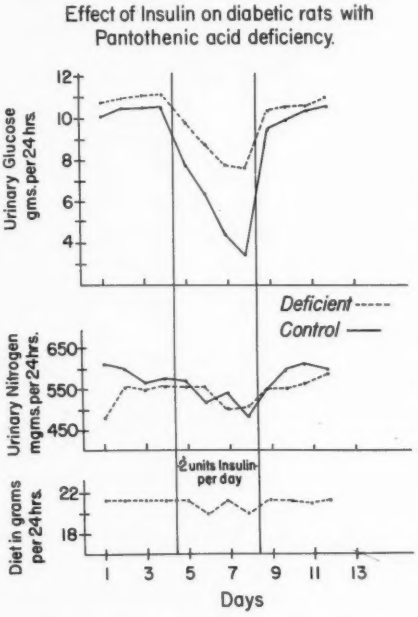
Effect of Insulin on diabetic rats with Pantothenic acid deficiency.
Urinary Glucose gms. per 24 hrs.
Deficient
Control
Urinary Nitrogen mgms. per 24 hrs.
2 units Insulin per day
Diet in grams per 24 hrs.
Days

FIG. 3. Each curve is an average of 4 diabetic rats. Day 1 of this experiment is after 96 days on the deficient and control diets.

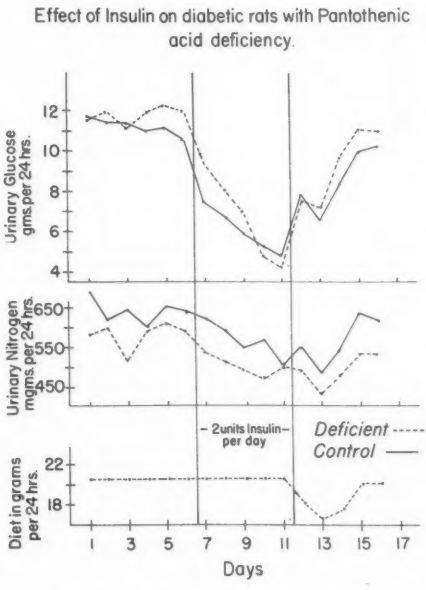
Effect of Insulin on diabetic rats with Pantothenic acid deficiency.
Urinary Glucose gms. per 24 hrs.
Urinary Nitrogen mgms. per 24 hrs.
2 units Insulin per day
Deficient
Control
Diet in grams per 24 hrs.
Days

FIG. 4. Each curve is an average of 5 diabetic rats. Day 1 of this experiment is after 125 days on the deficient and control diets.

The third group of diabetic animals, 4 deficient and 4 pairfed controls, was injected with protamine zinc insulin for 4 days after 23 weeks (160 days) on the purified diets. The data are shown in Figure 5. The pairfed control animals had a decrease in glycosuria from the average preinjection level of 10.3 to 4.5 gm. during the injection period (56% decrease). In contrast the deficient animals showed an increased sensitivity to insulin, exhibiting a decrease of approximately 83% in glycosuria, from the average preinjection level of 10.9 to 1.9 gm. This difference in glycosuria during the injection period is significant, the p value being less than 0.02. In this experiment (Fig. 5) and in the preceding one (Fig. 4) the nitrogen-sparing effect of insulin is clearly evident, *i.e.*, there is a lowering of the

urinary nitrogen during the period of insulin treatment. The degree of nitrogen sparing in the deficient animals did not differ from that of the pairfed control animals. This is in contrast to the resistance to the nitrogen-retaining effects of growth hormone observed in pantothenic acid deficiency (Hazelwood, Bennett, and Nelson, 1955).

Effect of ACTH in diabetic rats with pantothenic acid deficiency. Ten

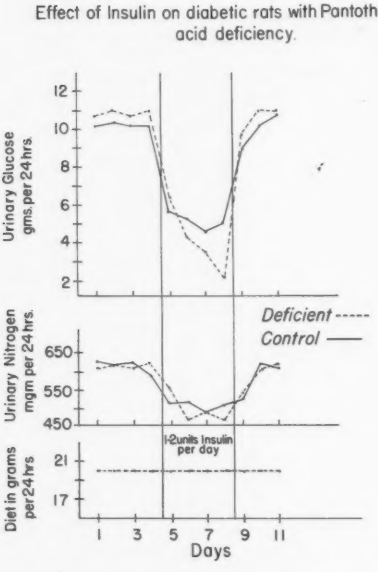
Effect of Insulin on diabetic rats with Pantothenic acid deficiency.
Urinary Glucose gms. per 24 hrs.
Urinary Nitrogen mgm per 24 hrs.
Deficient
Control
1-2units insulin per day
Diet in grams per 24 hrs
Days

FIG. 5. Each curve is an average of 4 diabetic rats. Day I of this experiment is after 156 days on the deficient and control diets. Three of the 4 deficient rats (and their pairfed mates) received 2 units of insulin per day. The remaining pair received 1 unit because of the low pretreatment glycosuria of the deficient animal.

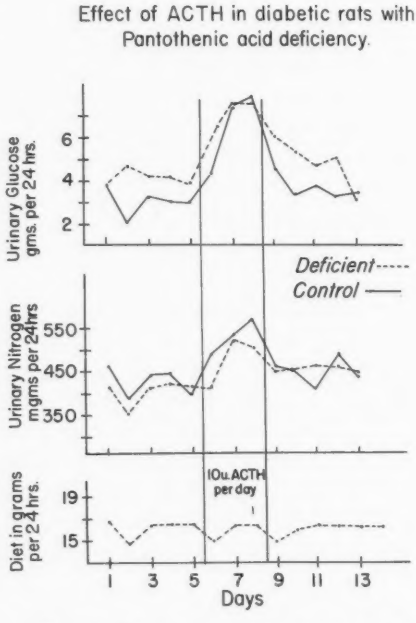
Effect of ACTH in diabetic rats with Pantothenic acid deficiency.
Urinary Glucose gms. per 24 hrs.
Deficient
Control
Urinary Nitrogen mgms per 24hrs
10u. ACTH per day
Diet in grams per 24 hrs.
Days

FIG. 6. Each curve is an average of 5 diabetic rats. Day 1 of this experiment is after 135 days on the deficient and control diets. Daily insulin injection was given as described in the text.

diabetic animals pairfed on the deficient and control diets for 140 days were used in this experiment. As these animals were severely diabetic, excreting an average of 9–10 gm. of glucose per day, it was thought unlikely that ACTH would produce a further detectable enhancement of this marked glycosuria. Therefore, for ten days prior to ACTH injection insulin was administered daily at the time of feeding in amounts of 1–1½ units per rat per day. (The deficient animals received the smaller doses of insulin.) When this regimen achieved a fairly stable partial control of the diabetes 10 units of Wilson's ACTH was injected into each animal two

hours after each day's feeding for three days. The data from this experiment are presented in Figure 6. There was a marked rise in both glycosuria and nitrogen excretion in both groups of animals. Although the increase in glycosuria was less marked in the deficient animals than in the controls the difference in response was not statistically significant.

DISCUSSION

The data presented show that insulin sensitivity in either intact or partially depancreatized diabetic rats varied with the duration of the pantothenic acid deficiency. In intact animals, 8 weeks of deficiency resulted in some resistance to insulin, a finding consistent with adrenal cortical hyperfunction. The adrenal glands of the deficient animals were larger than those of pairfed controls. The paired adrenals of the deficient animals had an average weight of 36 mg. as compared with the 26 mg. adrenals of the controls. In contrast, 14 weeks of deficiency resulted in an increased sensitivity to insulin, a finding consistent with adrenal cortical hypofunction. The adrenals of these deficient animals were smaller than those of pairfed controls, averaging 35 mg. in comparison with 42 mg. This increased sensitivity to insulin is in agreement with that observed by Winters *et al.* (1952) after 5–6 weeks of acute pantothenic acid deficiency in younger animals.

This variability in response to exogenous insulin administration was confirmed by studies on diabetic rats. In diabetic rats, 14 weeks of deficiency resulted in some resistance to insulin, 19 weeks of deficiency in approximately the same response as that shown by pairfed controls, and 23 weeks of deficiency in an increased sensitivity to insulin. In fact, the diabetic animals with severe pantothenic acid deficiency (140 days or more on the deficient diet) reacted so readily to insulin that several died in convulsive seizures, presumably due to hypoglycemic shock. Although the adrenals of such animals may be hypofunctional, they cannot be considered as being totally unresponsive to ACTH, as one group responded to ACTH (Wilson) with increased glycosuria and nitrogen excretion. The use of pairfed controls eliminated the factors of decreased food consumption or of other dietary deficiencies as causes for these changes in insulin sensitivity.

These physiological findings are in agreement with the conclusions drawn from the cytological studies on the adrenal cortex by Deane and McKibbin (1946) who observed an early phase of hyperactivity followed by a later phase of exhaustion and hypoactivity in rats subjected to this vitamin deficiency. The findings also indicate that variable results, depending on the severity of the deficiency, are to be expected in studies on adrenal cortical function during pantothenic acid deprivation. Rats with severe pantothenic acid deficiency have shown abnormalities consistent with adrenal cortical hypofunction, *e.g.* absence of lymphopenia and eosin-

openia following epinephrine or ACTH (Dumm *et al.*, 1949; Winters *et al.*, 1952; Hurley and Morgan, 1952); increased insulin sensitivity (Winters *et al.*, 1952); and decreased resistance to water intoxication (Gaunt *et al.*, 1946). Findings in rats with milder degrees of pantothenic acid deficiency have been consistent with either hyperactivity or normal adrenal cortical function, *e.g.* presence of lymphopenia and eosinopenia following epinephrine or ACTH (Ershoff *et al.*, 1953).

SUMMARY

The effect of varying degrees of pantothenic acid deficiency on insulin sensitivity has been studied in intact and in partially depancreatized diabetic male rats of the Long-Evans strain. In intact animals 8 weeks of deficiency beginning at 30 days of age resulted in some resistance to insulin whereas 14 weeks of deficiency resulted in increased sensitivity to insulin as compared with the sensitivity of pairfed controls.

The degree of glycosuria of diabetic rats during the development of pantothenic acid deficiency (104 days) did not differ from that of their pairfed controls. Depancreatized diabetic animals, when placed on the vitamin deficient diet beginning at 60 days of age, showed varying degrees of insulin sensitivity, as judged by the effectiveness of insulin in reducing glycosuria. Fourteen weeks of deficiency resulted in resistance to insulin, 19 weeks in approximately a normal response to insulin, and 23 weeks in a marked increase in insulin sensitivity. Diabetic animals maintained on the deficient diet for 140 days still responded to ACTH by an increase in glycosuria and nitrogen excretion. The use of pairfed controls eliminated the factors of inanition or other dietary deficiencies as causes for these changes in insulin sensitivity.

The results are interpreted as being consistent with adrenal cortical hyperfunction during the early phases of the vitamin deficiency followed by hypofunction in the later phases. This interpretation is in agreement with conclusions drawn from cytological studies on the adrenal cortex of rats subjected to this deficiency.

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THE INFLUENCE OF CHORIONIC GONADOTROPIN ON THE 3β-OL DEHYDROGENASE ACTIVITY OF TESTES AND ADRENALS[1]

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IT HAS been reported (Samuels *et al.*, 1951) that corpora lutea, placentae, interstitial cells of the testis, and the adrenal cortex contain an enzyme which would oxidize a 3β-hydroxy group in a steroid to a 3-ketone if the compound had an oxygen on C-17 or C-20. A wide range of other tissues, including the ovarian follicle, did not show this activity under the conditions studied. There did not seem to be any difference in specificity among the active tissues, since Δ^5-pregnen-3β-ol-20-one was converted to progesterone and dehydro*epi*androsterone was converted to Δ^4-androstene-3,4-dione by homogenates of either the testis, placenta or adrenal. It appeared, therefore, that this enzyme played a basic role in the formation of the nonbenzenoid steroid hormones and that the distinction in the final product from each gland depended on other enzymic reactions.

Since the output of steroid hormones by all of the tissues which contain this enzyme is dependent on tropic hormones, experiments were carried out to determine the effects of removal of the gland and of the introduction of a gonadotropin on the 3β-ol dehydrogenase of two of the tissues, the testis and adrenal cortex.

METHODS

Male rats of the Sprague-Dawley strain were hypophysectomized at 65–75 days of age by the parapharyngeal approach. They were maintained thereafter on a diet of bread, milk and hamburger, with periodic additions of carrots and orange juice, until used for the experimental procedure. Litter mates of these animals were also maintained intact to serve as controls on the effect of hypophysectomy. At selected intervals after removal of the hypophysis, groups of hypophysectomized and intact animals were decapitated, and the adrenals and testes removed, weighed and homogenized in cold buffer solution for immediate assay of the 3β-ol dehydrogenase.

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Two series of hypophysectomized rats were injected with human chorionic gonadotropin[3] and sacrificed at definite intervals thereafter. In one series the injections began 48 days after the operation, while there was an interval of 58 days in the other. The dosage of chorionic gonadotropin used was 100 I.U. per day injected subcutaneously In those studies in which the tissues were investigated more than 24 hours after the initial injection of chorionic gonadotropin, additional injections of 100 I.U. were given at the same time each day until the rats were sacrificed.

The 3β-ol dehydrogenase activity was determined using Δ^5-pregnen-3β-ol-20-one as substrate. The tissues were homogenized in a mixture of equal volumes of bovine serum and Krebs phosphate buffer containing 0.04 M niacin and 0.4 μM diphosphopyridine nucleotide (DPN). The substrate was dissolved in 0.2 ml. of propylene glycol, a volume of homogenate equivalent to a specified amount of tissue added, and the total volume made up to 20 ml. with a serum-buffer mixture. In nearly all cases at least two tissue concentrations were used. The testes homogenates were incubated for 3 hours at 33° C, approximately the temperature in the scrotum, while the adrenal homogenates were incubated for the same time at 37.5° C. Enzymic action was stopped by boiling and the contents of the incubation flasks were extracted and analyzed as previously described (Beyer and Samuels, 1955). In all cases aliquots of the homogenates incubated without the presence of substrate were extracted and used as blanks for the spectrophotometric analysis. In each series known amounts of progesterone were added to homogenate, boiled immediately and carried through the analysis to determine the adequacy of the recovery. This was always over 85% and in most series over 90%. The enzymic activity of the tissues was calculated in units. A unit of enzyme activity is that required to oxidize 0.1 μmole of Δ^5-pregnen-3β-ol-20-one in 3 hours under the conditions described.

In some experiments attempts were made to see whether chorionic gonadotropin could influence the 3β-ol dehydrogenase activity when incubated directly with tissue slices or homogenates. Since various conditions of incubation were used, these will be described in connection with the results.

RESULTS

Effects of hypophysectomy

Immediately after hypophysectomy there appeared to be an increase in the 3β-ol dehydrogenase activity of the testes, both per testis and per unit weight (Fig. 1). By 12 hours after the operation, however, the values were below those of the intact rats. Both values fell for the next 3–5 days. Thereafter the activity per testis decreased more gradually, the change between the 11th and 58th days being only from 34% to 22% of normal. About the 5th day the activity per unit weight of testis passed through a minimum and then increased until by the 58th day it was well over twice that of normal testicular tissue. This reversal in enzyme concentration was apparently due to the relatively more rapid atrophy of the interstitial cells compared with the seminiferous epithelium.

The 3β-ol dehydrogenase activity of the adrenals also fell after hypophysectomy (Fig. 2). The total activity per adrenal fell rather rapidly during the first 10 days to less than 40% of the normal controls. At about

[3] A.P.L. Chorionic Gonadotropin was generously supplied by Ayerst, McKenna and Harrison, Limited.

the 10th day the rate of change decreased but the activity continued to fall until at the end of 58 days it was only 16% of that of the adrenals of the normal controls. The change per unit weight was not nearly so marked, the activity on this basis only falling to 75% of the normal by the 19th

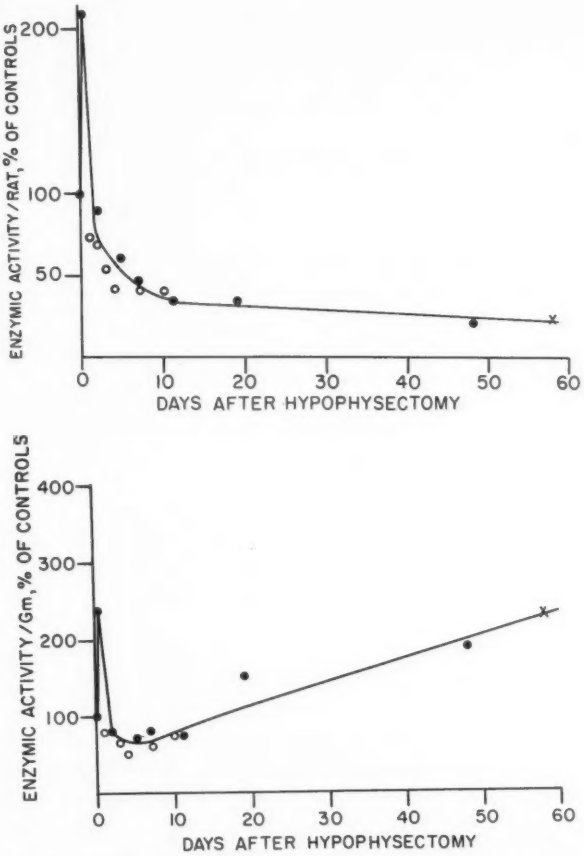

FIG. 1. Variation in the steroid 3β-ol dehydrogenase activity of the testes of mature rats with time after hypophysectomy. The enzymic activity of the testes of intact litter mates is taken as 100 per cent. Upper graph shows change in total activity of the two testes; lower graph shows change per unit weight of testis.

day, and to 67% of normal by the 58th day. Since the entire adrenal gland was homogenized for these measurements, and since only the adrenal cortex undergoes marked atrophy after hypophysectomy, the enzymic activity, which is largely, if not entirely, confined to the cortex (Samuels, 1953), probably remained at least as high per unit weight of cortex as in the normal gland.

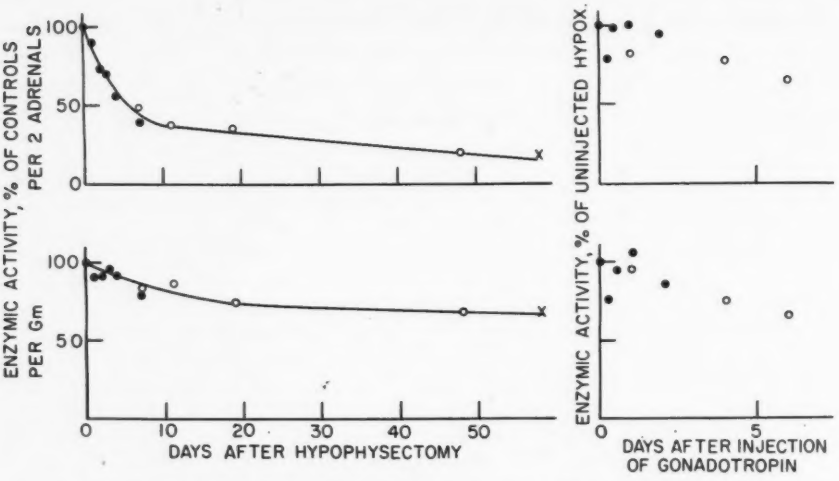

FIG. 2. Variation in the steroid 3β-ol dehydrogenase activity of the adrenals of mature male rats with time after hypophysectomy, and after injection of chorionic gonadotropin. The enzymic activity of the adrenals of intact litter mates is taken as 100 per cent. Upper graphs are on the basis of total activity in two adrenals; lower graphs are on the basis of unit weight of adrenals.

Effects of injections of chorionic gonadotropin

The effect of injection of 100 I.U. chorionic gonadotropin per day on the 3β-ol dehydrogenase activity of the testes of hypophysectomized rats is shown in Figure 3. Two series of animals were studied, and it can be seen

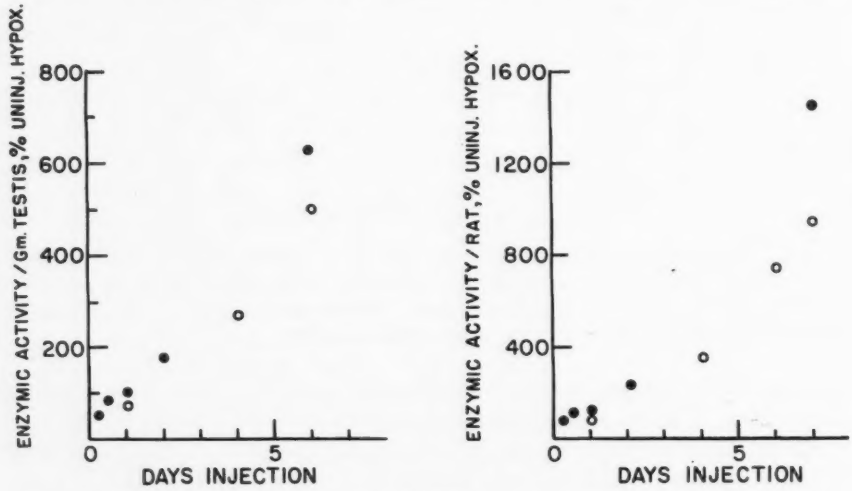

FIG. 3. Effect of injection of 100 I.U. chorionic gonadotropin on the steroid 3β-ol dehydrogenase activity of the testes of hypophysectomized rats.

that there was some difference in the response of the two groups. (This was also true in the changes after hypophysectomy but, as can be seen in Figure 1, these were small in comparison with the general effect). The activity, both per unit testis weight and per testis, increased rapidly in both series. Within 4 days the activity per testis exceeded that of the intact controls, and by 6 days it was more than double this value. The activity per unit weight, which in rats hypophysectomized for this length of time would already be about double that of testes of intact rats of the same age, continued to increase because the chorionic gonadotropin had

TABLE 1. EFFECT OF CHORIONIC GONADOTROPIN IN VITRO ON 3β-ol DEHYDROGENASE ACTIVITY OF TESTES FROM HYPOPHYSECTOMIZED RATS

No. rats	Preparation	Incubated with C.G.*	Injected with C.G.†	Preincubated		DPN added		3β-ol dehydrogenase	
				−C.G.	+C.G.	Preinc.	Inc.	u./gm.	u./rat
3	Slices	−	−	−	−	−	+	26.3 (20.0–32.8)	(12.0–23.2)
3	Slices	−	+	−	−	−	+	68.5 (48.0–82.0)	69.5 (48.0–82.0)
2	Slices	+	−	−	−	−	+	28.8 (20.4–37.1)	19.4 (14.7–24.1)
3	Slices	+	−	+	−	+	+	17.7 (15.9–20.9)	13.5 (11.1–15.3)
4	Slices	+	−	−	+	+	+	18.2 (15.9–19.5)	13.1 (11.0–14.6)
7	Homogenate	−	−	−	−	−	+	11.1 (6.5–20.0)	
5	Homogenate	−	+	−	−	−	+	63.0 (43.0–94.0)	
3	Homogenate	+	−	−	+	+		6.7 (5.9– 7.2)	
3	Homogenate	−	−	+	−	+		6.6 (4.2– 9.0)	

* 100 I.U. Chorionic Gonadotropin (Ayerst APL) added per flask.
† 100 I.U. Chorionic Gonadotropin (Ayerst APL) injected subcutaneously daily for 6 days.

more effect on the interstitial cells than on seminiferous epithelium. Thus the testes of hypophysectomized rats so treated were many fold more active sources of the enzyme per unit mass than those of intact adults.

During the period when the 3β-ol dehydrogenase activity was increasing so rapidly in the testes under the influence of chorionic gonadotropin, that of the adrenals seemed actually to fall at a rate somewhat greater than in untreated hypophysectomized rats this long after operation (Fig. 2). It seems, therefore, that the action of the gonadotropin was not a general one on steroid 3β-ol dehydrogenases.

Effect of incubation of testis slices and homogenates with chorionic gonadotropin

In Table 1 the results of incubation of various preparations of testis tissue with chorionic gonadotropin have been summarized. In no case was there any evidence of increased enzymic activity, either when the gonado-

tropin was preincubated with the testis tissue or when it was introduced simultaneously with the substrate. This was not due to lack of DPN since in some incubations this was introduced during the preincubation with the gonadotropin and in others was introduced at the same time as substrate. If the preincubation with the tropic hormone had any influence it was to lower enzymic activity. In view of these results it would appear that under the conditions studied, the chorionic gonadotropin does not directly activate this enzyme system.

DISCUSSION

The results of these experiments indicate that the steroid 3β-ol dehydrogenase is primarily a constituent of the Leydig cells and not of the germinal epithelium. The effect of chorionic gonadotropin on the enzyme is not that of activation; instead, it apparently affects, directly or indirectly, synthesis of the enzyme.

The immediate rise in 3β-ol dehydrogenase activity after hypophysectomy probably is the result of a large output of gonadotropin during manipulation of the pituitary gland; the minimum through which the concentration of 3β-ol dehydrogenase passes between the 5th and 10th day after hypophysectomy and the subsequent rise to values above that of the normal testis, are probably attributable to the more rapid atrophy of the Leydig cells compared to that of the germinal epithelium. This hypothesis involves the assumption that the enzyme is limited to the Leydig cells. The assumption and the hypothesis are supported by recent studies of Steinberger and Nelson (1955) on the changes in concentration of testicular hyaluronidase. The activity of this enzyme had been previously shown to be related to the functional activity of the germinal epithelium (Sprunt *et al.*, 1939; Leonard *et al.*, 1946, 1948). By the 6th day after hypophysectomy the Leydig cells showed atrophy while no noticeable changes had occurred in the germinal tissues. Steinberger and Nelson found that the hyaluronidase activity per unit weight of testis did not fall significantly until between the 6th and 10th days after operation; the drop was then rapid until the 20th day, after which it tended to level off. The period during which the germinal epithelium and hyaluronidase activity were maintained corresponds well with the period of rapid fall in 3β-ol dehydrogenase concentration; the period of rapid fall in hyaluronidase activity, associated with disintegration of the seminiferous tubules, is also the period during which we observed a rapid rise in concentration of 3β-ol dehydrogenase, even though the total activity per testis continued to decrease slowly. From the 20th day on, the activities of both hyaluronidase and 3β-ol dehydrogenase followed the course which might be expected if both Leydig cells and seminiferous epithelium continued to atrophy slowly. Thus the relative changes in activity of the two enzymes fit well with the concept that the hyaluronidase is associated with the larger

mass of tissue, the seminiferous epithelium, and the 3β-ol dehydrogenase with the cytoplasm of the Leydig cells.

In their histological examination, Steinberger and Nelson state that by the 20th day of hypophysectomy the Leydig cells had disappeared. That this apparent disappearance is not truly a complete regression to connective tissue is indicated by the persistence of total 3β-ol dehydrogenase activity at low levels (20% of normal) for as long as 59 days after the operation. Since other tissues having relatively large numbers of fibroblasts do not show this enzymic activity (Samuels *et al.*, 1951) it would seem that the atrophic Leydig cells remain distinct chemically even though they can no longer be distinguished histologically from fibroblasts.

Normal levels of gonadotropin in the young adult male rat apparently do not stimulate the Leydig cells maximally; from the low levels of 3β-ol dehydrogenase activity after 58 days of hypophysectomy the total could be raised to more than twice normal by 6 days of treatment with human chorionic gonadotropin.

The gonadotropin had little effect on the germinal epithelium since there was a rapid increase in the concentration of the enzyme, already higher than in normal testes. If the tropic hormone had stimulated the growth of the seminiferous epithelium the increase in concentration of the enzyme would not have been so rapid or so marked in spite of the greater total amount. This very high concentration indicates that such stimulated testes of hypophysectomized animals should be a good source for isolation of the enzyme.

The increase in 3β-ol dehydrogenase activity after injection of chorionic gonadotropin seems to be significant within 48 hours, but neither the rate of increase nor the results of the *in vitro* addition of the gonadotropin indicate a direct activating influence on the enzyme already present. The picture is that to be expected if synthesis of the enzyme were stimulated. Yet the experiments of Brady (1951) indicate that chorionic gonadotropin introduced *in vitro* increases the incorporation of C^{14}-acetate into testosterone in the presence of testes slices. This resembles the apparent action of adrenocorticotropic hormone on some early stage in the synthesis of adrenal steroids (Stone and Hechter, 1954). It seems most probable, therefore, that the action of the tropic hormone is directly on some other step in androgen synthesis, and that the increase in 3β-ol dehydrogenase activity is an indirect response to this activation. According to this hypothesis the enlargement of glands, such as the interstitial tissue of the testis or the cortex of the adrenal, which follows the injection of the appropriate pituitary hormones is a form of adaptive enzyme synthesis. It may be, however, that the effects on activation of a specific enzyme and on protein synthesis in the cells may be independent actions of the tropic hormones, both leading to increased hormone output. The present experiments do not distinguish between these two possibilities.

SUMMARY

After a temporary increase in activity, the steroid 3β-ol dehydrogenase activity of the testis fell rapidly after hypophysectomy. On the basis of total activity per testis the activity dropped rapidly during the first 5–6 days and then at a decreasing rate over the 58-day period studied. The activity per unit mass of testis reached a minimum at about 5 days after the operation and then gradually rose to concentrations well above normal by the 20th day. This high concentration was maintained throughout the 58 days studied.

On the injection of chorionic gonadotropin the total steroid 3β-ol dehydrogenase activity of the atrophic testes rose to values more than twice normal. Since there was no simultaneous stimulation of the seminiferous epithelium, the already elevated concentration of the enzyme rose to very high levels.

While the steroid 3β-ol dehydrogenase activity of the adrenal glands of the hypophysectomized rats decreased with atrophy of the glands, the activity per unit mass did not drop significantly. On injection of chorionic gonadotropin there was no increase in the activity of this enzyme in the adrenals as there was in the testes.

Various systems of incubation of the atrophic testes of hypophysectomized rats with chorionic gonadotropin *in vitro* did not lead to any increase in 3β-ol dehydrogenase activity.

It is concluded that chorionic gonadotropin caused an increase in steroid 3β-ol dehydrogenase activity by increasing the synthesis of Leydig cell protein rather than through activation of existing enzyme. This was probably an indirect result of activation of another enzymic step by the tropic hormone, an "adaptive" effect. The possibility is not ruled out, however, that the effect on Leydig cell protein synthesis is a slower independent action of the gonadotropin.

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THE RELATIVE IMPORTANCE OF ANDROGEN AND ESTROGEN IN THE SELECTIVE UPTAKE OF Zn^{65} BY THE DORSOLATERAL PROSTATE OF THE RAT

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THE striking ability of the dorsolateral prostate of the mature rat to concentrate administered Zn^{65} (Gunn, Gould *et al.*, 1955) led to a study of the endocrine factors possibly controlling the selective uptake of Zn^{65} by this accessory sex gland.

EXPERIMENTAL PROCEDURE

The uptake of Zn^{65} by the dorsolateral prostate was studied in male Wistar rats of various age groups and in various states of induced hormonal imbalance. The hormones were administered for six days prior to the initiation of the Zn^{65} studies. All experiments were done between the months of July and October, and control animals were run with each experiment.

Studies in the Mature Rat. One hundred mature male Wistar rats, 15–17 weeks of age (350–400 gm.), were used in these studies. A total of 20 rats were used as intact controls, and the remaining animals were divided into the following experimental groups. From 4–7 animals were used for each dosage listed.

(1) Castrate controls.

(2) Castrates administered daily replacement of testosterone[1] in doses of 25, 50 and 100 μg.

(3) Intact rats administered 50 μg. of testosterone daily.

(4) Intact rats administered estradiol[2] daily in doses of 0.1, 0.5, 1.0, 1.5, 10 and 100 μg.

(5) Castrates administered daily replacement of estradiol in doses of 0.1, 1.0, 10 and 100 μg.

Studies in the Immature Rat. Sixty immature male Wistar rats, 6 weeks of age (100–150 gm.), were used in these studies. A total of 10 rats were used as intact controls, and the remaining animals were divided into the following experimental groups. From 5–10 animals were used for each dosage listed.

(1) Intact rats administered testosterone daily in doses of 50 and 100 μg.

(2) Intact rats administered 2 units of gonadotropnin[3] daily

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[1] Generously supplied by the Schering Corporation as testosterone propionate in oil, administered in 0.2 ml. subcutaneous injections.

[2] Generously supplied by the Schering Corporation as estradiol benzoate in oil, administered in 0.2 ml. subcutaneous injections.

[3] Generously supplied by Parke, Davis and Company as chorionic gonadotrophin in saline, administered in 0.2 ml. subcutaneous injections.

(3) Intact rats administered estradiol daily in doses of 0.1 and 10 μg.
(4) Castrate controls
(5) Castrates administered daily replacement of 50 μg. of testosterone
(6) Castrates administered daily replacement of 10 μg. of estradiol.

Studies in Rats of Various Ages. In addition to the animals listed above, 30 male Wistar rats between 6 and 15 weeks of age were used to determine the degree of uptake of Zn^{65} by the dorsolateral prostate at various ages.

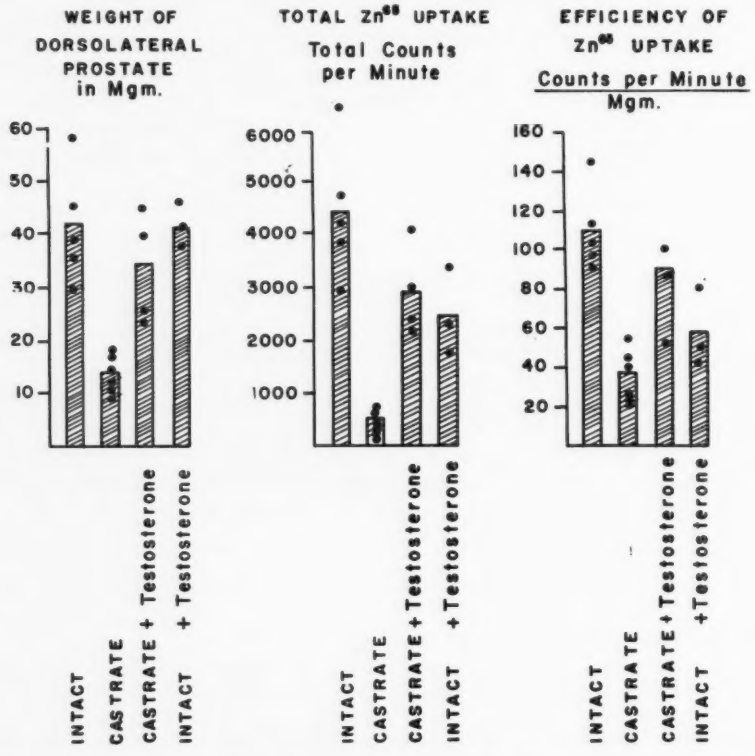

FIG. 1. The effect of castration and testosterone administration on the weight, total Zn^{65} uptake, and efficiency of Zn^{65} uptake of the dorsolateral prostate of the mature rat: (1) intact control rat, (2) castrate rat (one week post-castration), (3) castrate rat administered daily replacement of 50 μg. of testosterone propionate for one week, and (4) intact rat administered daily doses of 50 μg. of testosterone propionate for one week.

Zn^{65} Studies

Studies were begun on the uptake of Zn^{65} by the dorsolateral prostate on the sixth day post-castration, or on the sixth day after hormone administration was initiated. The techniques used in the Zn^{65} studies were the same as those outlined in a previous publication (Gunn, Gould *et al*, 1955) with the exception that radioactivity counts and calculations were based on dry weight of the dorsolateral prostate.

Zn^{65} (0.4 μc./gm.)[4] was administered by intracardiac injection, and 24 hours later

the rats were chloroformed and bled to remove most of the blood from the tissues. The dorsolateral lobes of the prostate were dissected out, placed in metal planchets, dried and weighed. Radioactivity was measured by an end-window Geiger-Muller counting tube in conjunction with a decade scaler. The experimental results are expressed in the Figures as Weight of Dorsolateral Prostate in Mg. (Dry Weight), Total Zn^{65} Uptake of the Gland (Total Counts per Minute), and Efficiency of Zn^{65} Uptake (Counts per Minute per Mgm. of Tissue).

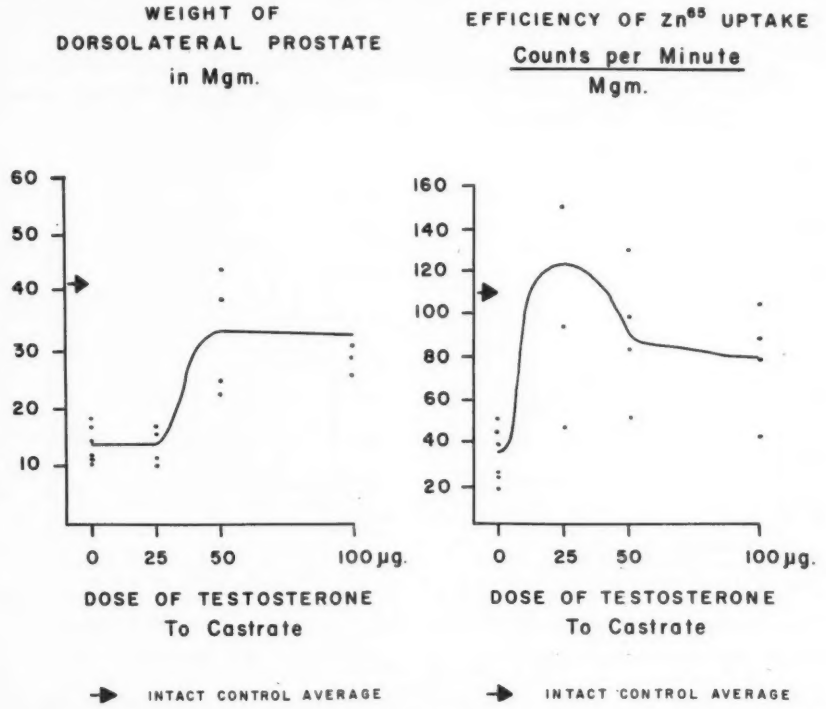

FIG. 2. The effect of various doses of testosterone replacement on the weight and Zn^{65} uptake of the dorsolateral prostate of the castrate mature rat: daily administration of 25, 50 and 100 µg. of testosterone propionate for one week.

RESULTS

The effect of castration and testosterone administration on the Zn^{65} uptake of the dorsolateral prostate of the mature rat (Fig. 1). One week following castration there was a marked diminution in both the size and total Zn^{65} uptake of the dorsolateral prostate. The fall in Zn^{65} uptake was more than could be accounted for by the diminished size of the gland, thus indicating

[4] Zn^{65} was purchased from Carbide and Carbon Chemicals Co. as $Zn^{65}Cl_2$ in HCl solution with a specific activity of 95–336 mc./gm. The solution was diluted with 0.85% NaCl containing sufficient NaOH to neutralize the acid present. The dilution was made so that the administered dose was contained in less than 0.4 ml.

that the efficiency of the residual glandular tissue to concentrate Zn^{65} also was impaired. With the daily administration of 50 μg. of testosterone propionate to the castrate, there was far less diminution in the size of the dorsolateral prostate, and the efficiency of the gland to take up Zn^{65} was maintained near control levels. It is interesting that the administration of this same dose of androgen to the intact mature rat resulted in a depression of the Zn^{65} uptake without affecting the weight of the gland.

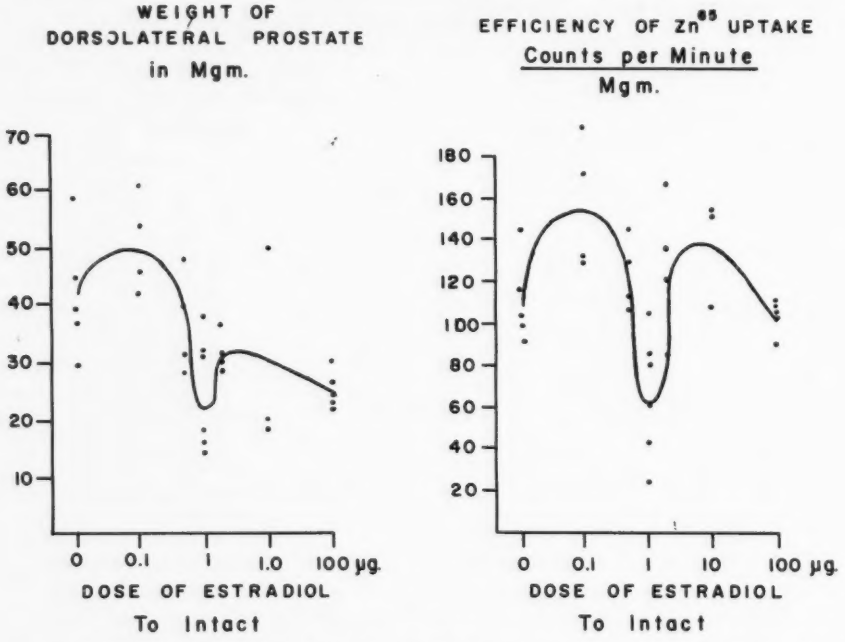

FIG. 3. The effect of various doses of estradiol benzoate on the weight and Zn^{65} uptake of the dorsolateral prostate of the intact mature rat: daily administration of 0.1, 0.5, 1.0, 1.5, 10 and 100 μg. of estradiol benzoate for one week.

The effect of various doses of testosterone in maintaining the Zn^{65} uptake of the dorsolateral prostate of the mature castrate rat (Fig. 2). The lowest dose of testosterone administered 25 μg., did not prevent the fall in weight of the dorsolateral prostate following castration. Nevertheless, this small dose of androgen was very effective in maintaining the capacity of the residual gland to concentrate Zn^{65}. Both 50 and 100 μg. of testosterone prevented the striking fall in weight and efficiency of Zn^{65} uptake of the dorsolateral prostate. It is interesting that, with regard to the efficiency of the gland, the higher dose of testosterone (100 μg.) was less effective on the average than the lower dose (25 μg.).

The effect of various doses of estradiol on the Zn^{65} uptake of the dorsolateral

prostate of the intact mature rat (Fig. 3). Of the various doses of estradiol benzoate tested between 0.1 and 100 μg., only one dose, 1 μg., was found to depress appreciably the efficiency of Zn^{65} uptake of the dorsolateral prostate. The range of depression was an extremely narrow one as shown by the fact that doses of 0.5 and 1.5 μg. failed to affect this function. Although all doses of estradiol tested from 1 to 100 μg. decreased the size of the dorsolateral prostate, the efficiency of the residual tissue to concentrate Zn^{65} was not depressed even with doses as high as 10 and 100 μg. At the

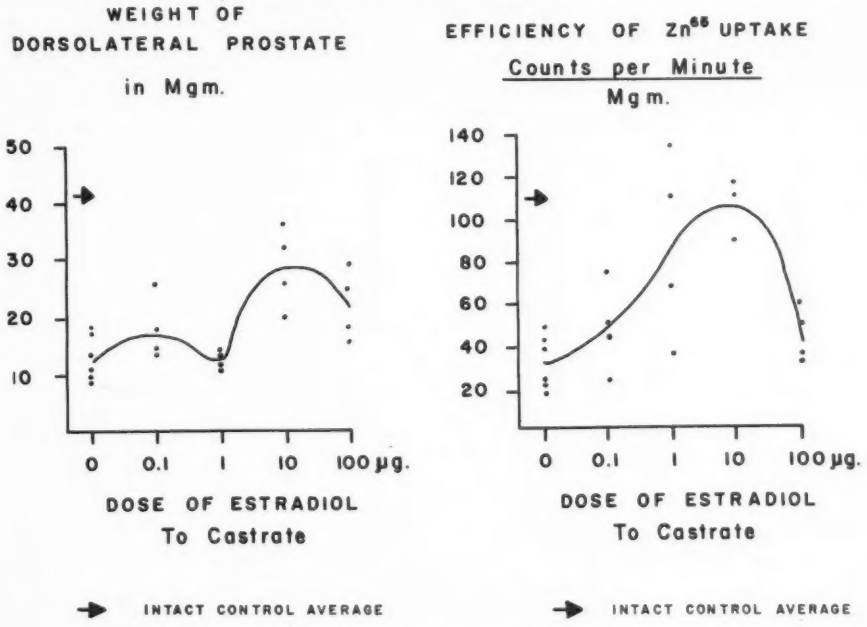

FIG. 4. The effect of various doses of estradiol replacement on the weight and Zn^{65} uptake of the dorsolateral prostate of the castrate mature rat: daily administration of 0.1, 1.0, 10 and 100 μg. of estradiol benzoate for one week.

lowest dose tested, 0.1 μg., there was a slight increase in both the weight and efficiency of the gland to take up Zn^{65}.

The effect of various doses of estradiol in maintaining the Zn^{65} uptake of the dorsolateral prostate of the mature castrate rat (Fig. 4). Estrogen was very effective in preventing the marked decrease in efficiency of Zn^{65} uptake by the dorsolateral prostate following castration. Estrogen was more effective as the dose was increased up to 10 μg., a dose which afforded complete replacement to the castrate as far as the efficiency of Zn^{65} uptake was concerned. However, increasing the dose to 100 μg. depressed the efficiency. Estrogen also had a slight effect in maintaining the weight of the dorsolateral prostate following castration.

The Zn^{65} uptake of the dorsolateral prostate of the rat at various ages (Fig. 5). The capacity of the dorsolateral prostate to concentrate Zn^{65} increases as the age of the animal increases, with maximum efficiency to take up Zn^{65} being reached at about 12 to 15 weeks of age.

The comparative effects of androgen, estrogen and gonadotrophin on the Zn^{65} uptake of the dorsolateral prostate of the intact immature rat (Fig. 6).

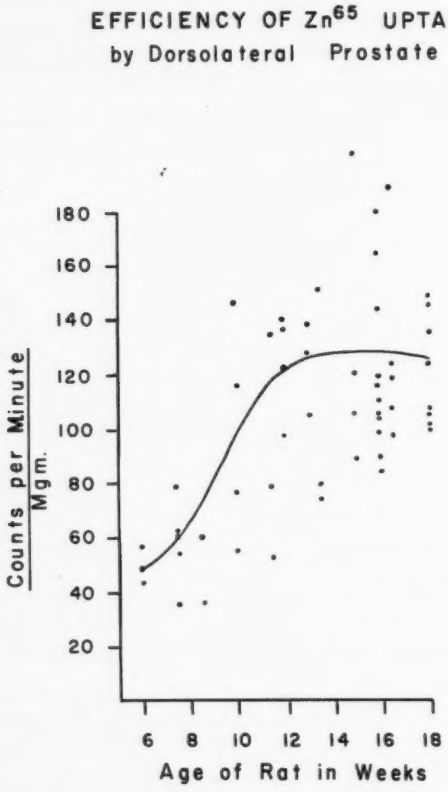

FIG. 5. The efficiency of Zn^{65} uptake of the dorsolateral prostate of the rat at various ages, from 6 to 18 weeks of age.

Although testosterone and gonadotrophin slightly enhanced the growth of the dorsolateral prostate of the immature rat, neither hormone had any effect on the capacity of the gland to take up Zn^{65}. However, 10 μg. of estradiol, which depressed the growth of the dorsolateral prostate, actually raised the efficiency of the immature gland to concentrate Zn^{65} to a mature level. A low dose of 0.1 μg. of estradiol had no significant effect on either the weight or efficiency of the gland (results not shown in the Figure).

The comparative effects of androgen and estrogen on the Zn^{65} uptake of the dorsolateral prostate of the immature castrate rat (Fig. 6). Androgen was able to prevent the slight fall in weight and efficiency of the immature dorsolateral prostate to concentrate Zn^{65} as a result of castration. In contrast, estrogen to the castrate, although unable to maintain the weight of the gland at the intact immature level, actually raised the efficiency to concentrate Zn^{65} to the level of a mature gland.

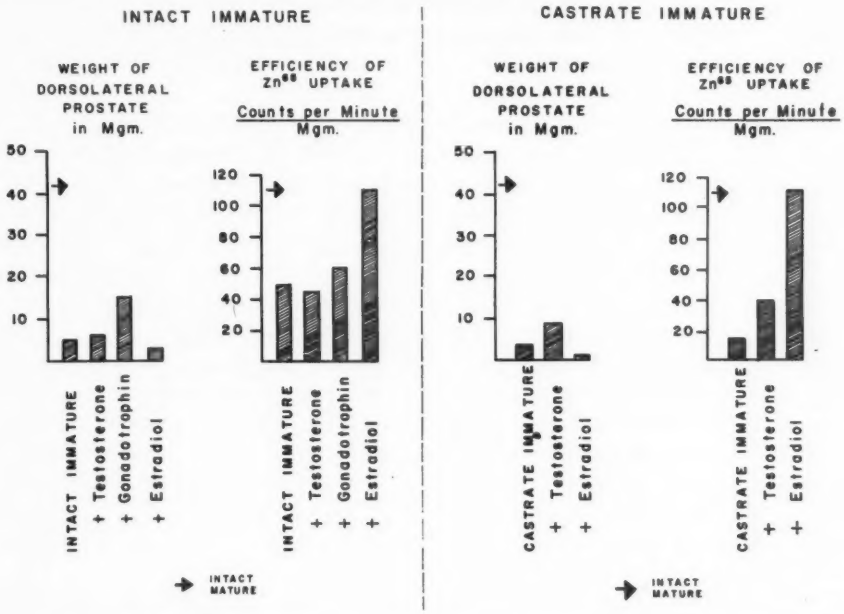

FIG. 6. The effect of testosterone, gonadotrophin and estradiol on the weight and Zn^{65} uptake of the dorsolateral prostate of the intact immature rat, and of testosterone and estradiol replacement on the weight and Zn^{65} uptake of the dorsolateral prostate of the castrate immature rat: (1) intact control rat, (2) intact rat administered 50 μg. of testosterone propionate, (3) 2 units of chorionic gonadotrophin, and (4) 10 μg. of estradiol benzoate daily for one week, (5) castrate rat (one week post-castration), (6) castrate rat administered replacement of 50 μg. of testosterone propionate, and (7) 10 μg. of estradiol benzoate daily for one week.

DISCUSSION

Up to the present time the only measure of the functional capacity of the dorsolateral prostate has been the concentration of fructose in that gland (Mann and Parsons, 1950). Recently it has been shown that there is a selective uptake of administered Zn^{65} by the dorsolateral prostate of the rat (Gunn, Gould *et al.*, 1955), and that a high proportion of the radioactive zinc in the gland is present in the secretion (Gunn and Gould, un-

published data). Thus it appears that the degree of uptake of Zn^{65} by the dorsolateral prostate is an indication of the functional state of that organ.

Classic studies in endocrinology have shown that androgen is necessary for the mature growth and function of the accessory sex glands of the male. The role of estrogen in the male appears to be more complex. Estrogen reduces the secretory capacity of the male accessory organs with a resultant shrinkage of the glands, and in certain doses enhances the effects of androgen on both weight and functional activity (Burrows, 1949). The relative importance of both androgen and estrogen in the selective uptake of Zn^{65} by the dorsolateral prostate has been investigated in the studies reported in this paper.

Mann and Parsons (1950) have shown that the weight and fructose content of the dorsolateral prostate were depressed as a result of castration, and that the administration of increasing doses of androgen to the castrate resulted in proportionate increases in both the weight and fructose production of the gland. Similarly, the results presented in this paper show that the function of the dorsolateral prostate, as reflected in its capacity to take up Zn^{65}, was depressed by castration, and that this marked depression was prevented by the administration of suitable doses of androgen. However, the maintenance of efficiency of Zn^{65} uptake in the castrate in response to various doses of testosterone did not parallel the maintenance of glandular size. For example, it was found that a dose of testosterone which was insufficient to maintain the size of the gland, was ample to maintain the efficiency of the cells to concentrate Zn^{65} at control levels. Furthermore, increasing the dose of testosterone in the castrate beyond a certain range necessary for maintenance of both weight and function, tended to depress the efficiency of the gland to take up Zn^{65}.

Similar depressions in this function were noted when exogenous androgen was administered to the intact mature rat and to the mature hypophysectomized rat maintained on gonadotrophin (Gunn and Gould, unpublished data). In none of these instances, however, did the excess androgen alter the size of the gland. These results showed that excess androgen was interfering with some factor essential for maintenance of the efficiency of the dorsolateral prostate to concentrate Zn^{65} at optimal levels. The results also indicated that this interference was neither at the pituitary nor at the testicular level, but may have occurred at the level of the dorsolateral prostate itself, or may have been the result of neutralization by androgen of some other essential factor.

Reviewing the effects of estrogen in these Zn^{65} studies, it was surprising that in the wide span of dosage tested there was only a narrow range of dosage, around 1.0 μg., which depressed the Zn^{65} uptake of the dorsolateral prostate. In fact, at the lower dose tested, 0.1 μg., there was actually an increase in efficiency of Zn^{65} uptake by the gland. The remarkable effects of estrogen in maintaining the efficiency of the dorsolateral prostate to

concentrate Zn^{65} in the castrate indicates that estrogen is very important in this glandular function.

Experiments in younger rats demonstrated that the dorsolateral prostate of the immature rat was unable to concentrate Zn^{65} to as remarkable a degree as that of the mature animal. The efficiency of the gland to concentrate Zn^{65} increased with increasing age, reaching a maximum at about 12 to 15 weeks of age. This linear relationship between age and capacity to concentrate administered Zn^{65} parallels closely the increase in natural zinc content of the gland with growth, as reported by Fischer, Tikkala and Mawson (1955).

The question was posed, what factors were lacking in the immature rat which prevented the dorsolateral prostate from taking up Zn^{65} to any remarkable degree. Although both androgen and gonadotrophin were able to enhance the growth of the dorsolateral prostate, neither hormone had any effect on the Zn^{65} function of the gland. It was indeed startling that estrogen, which reduced the size of the dorsolateral prostate, actually raised the efficiency of Zn^{65} uptake of the immature gland to a mature level. The full significance of estrogen was revealed in experiments in the immature castrate, where in the absence of the testes, estrogen, but not androgen, was able to bring the capacity of the dorsolateral prostate to concentrate Zn^{65} up to the level found in the intact, fully mature rat.

SUMMARY

It has been shown that the selective uptake of administered Zn^{65} by the dorsolateral prostate of the rat is influenced by both androgen and estrogen. The administration of suitable doses of androgen to the castrate mature rat prevented the striking fall in Zn^{65} uptake of the dorsolateral prostate as a result of the castration. However, the administration of exogenous androgen to the intact mature rat actually depressed the efficiency of the gland to concentrate Zn^{65}. Estrogen inhibited the Zn^{65} uptake of the dorsolateral prostate of the intact mature rat in only a very narrow range of dosage. On the other hand, in the mature castrate, estrogen in certain doses was equally as effective as androgen in maintaining the Zn^{65} uptake of the gland.

The dorsolateral prostate of the immature rat was unable to concentrate Zn^{65} to as remarkable a degree as that of the mature animal. The efficiency of Zn^{65} uptake of the dorsolateral prostate of the immature rat was not altered by the administration of either androgen or gonadotrophin. However, the efficiency of the immature gland to concentrate Zn^{65} was raised to a mature level by a suitable dose of estrogen. In the immature castrate estrogen, but not androgen, was able to bring the capacity of the dorsolateral prostate to concentrate Zn^{65} up to the level found in the intact, fully mature rat.

Acknowledgments

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ADRENOCORTICAL INFLUENCES ON DIPEPTIDASE ACTIVITY OF SURVIVING RAT DIAPHRAGM[1]

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THE attractive and oft-quoted hypothesis that hormonal influences in the organism are mediated through direct action on specific enzyme activities has been difficult to substantiate on any broad base (Dorfman and Goldsmith, 1951). The failure to establish such a relationship between adrenocortical function and intracellular peptidase activity provides a pertinent example. Though an over-all protein catabolic effect of corticosteroids is well documented, previous studies revealed that peptidase activity of rat (Schwartz and Engel, 1949) and human (Schwartz and Engel, 1950) serum as well as aqueous extracts and cell-free suspensions of rat tissue (Engel and Schwartz, 1951) is essentially uninfluenced by the administration of corticotropin or adrenal steroids. Despite these unencouraging results, the same line of inquiry has been pursued using *intact surviving* rat diaphragm (Schwartz, *et al.*, 1952) as a source of enzyme.

The studies reported here, therefore, were designed to bear on the question of whether the protein catabolic effect of adrenal steroids was achieved, at least in part, by provoking enhanced activity of specific proteolytic enzymes functioning within viable cells. Muscle was chosen as an appropriate tissue since it offers, quantitatively, the greatest bulk of mobilizable protein. The intracellular dipeptidase which quite specifically hydrolyzes glycylglycine was selected since it has been well characterized (Smith, 1948b) and responds strikingly to the influence of chemical activators and inhibitors. Its physiologic role, however, remains in question. Presumably, it hydrolyzes glycylglycine (and sarcosylglycine, its other known substrate) liberated from catabolized intracellular protein. However it is probable that this dipeptidase is involved, as well, in essentially synthetic (transpeptidation) reactions (Fruton, 1950). Indeed, Zamecnik and Frantz (1950) have demonstrated that a preparation of dipeptidase from hog intestinal mucosa does catalyze such reactions.

At any rate, when this problem is re-examined using intact surviving

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rat diaphragm as the enzyme source, unequivocal effects of the adrenal cortex on dipeptidase activity are demonstrable. In addition, some studies bearing on the possible mode of action of cortisone on dipeptidase activity are described.

MATERIALS AND METHODS

Experimental Procedure.—The procedure used is essentially that described previously (Schwartz, *et al.*, 1952) but certain simplifying modifications have been instituted without loss of accuracy or specificity.

Diaphragms are obtained from nembutal-anesthetized, exanguinated 150 to 250 gm. rats of the Vanderbilt strain which have been subjected to an 18-hour fast. After bisection, the hemidiaphragms are weighed and one, serving as a control, is immersed in 4 ml. of 0.0444N picric acid. The other hemidiaphragm is suspended in a 10 ml. Erlenmeyer flask containing 3 ml. of a medium made 0.34 m*M* with respect to glycine, as suggested by Christensen and Streicher (1949). The flask is partially immersed and shaken in a

TABLE 1. EFFECT OF GRINDING ON GLYCINE AND AMINO NITROGEN CONTENT OF RAT DIAPHRAGM

Grinding medium	Glycine (μM/gm. diaphragm)				Amino nitrogen* (μM/gm. diaphragm)			
	Control hemidiaphragm	Ground hemidiaphragm	Difference	P	Control hemidiaphragm	Ground hemidiaphragm	Difference	P
Picric acid	0.97 ± 0.09†	1.10 ± 0.05	+0.13 ± 0.05	<0.1 (4)	38.6 ± 0.4	40.2 ± 0.6	+1.6 ± 0.5	<0.02 (8)
Christensen's medium	0.89 ± 0.04	0.97 ± 0.05	+0.08 ± 0.03	<0.05 (4)	42.3 ± 0.4	43.4 ± 0.8	+1.1 ± 0.6	<0.2 (5)
Christensen's medium (postincubation with 42.8 mμ (GG)	6.35 ± 0.38	6.28 ± 0.36	−0.07 ± 0.10	>0.5 (4)				

* Determined by the photometric procedure of Moore and Stein (1948).
† Mean ± Standard Error.
Numbers in parentheses represent the number of animals used.

Warburg bath maintained at 37° C. A constant flow of a mixture of 95% oxygen-5% carbon dioxide is directed through each individual flask. The diaphragm is "washed" in this manner for 15 minutes and then transferred to a second similar flask for an additional 15 minutes. (It has been found that two of the four such washing periods originally described were dispensable.) The hemidiaphragm finally is transferred to a flask containing 3 ml. of the same medium containing no free glycine but supplemented with 42.8 mM/ml. of glycylglycine (GG). Incubation is usually continued for an additional 30 minutes. At the end of this hour of washing and incubation, the hemidiaphragm is immersed in 4 ml. of picric acid solution contained in a centrifuge tube. The incubation medium is quantitatively transferred to a 10 ml. volumetric flask and made up to volume with picric acid solution. After mixing well, the contents of the flask are centrifuged and the clear supernatant stored in the refrigerator prior to analysis for glycine content.

In previous studies, following the appropriate experimental manipulation, hemidiaphragms were ground in picric acid solution contained in a mortar before analysis for glycine (Schwartz, *et al.*, 1952) or αamino nitrogen (Schwartz, 1953a). It was noted that results remained essentially unchanged despite the fact that constantly waning enthusiasm for this procedure led, by imperceptible degrees, to somewhat superficial and incomplete maceration. For this reason, the influence of grinding the diaphragms on the extraction of glycine and free αamino nitrogen was investigated. Results are recorded in Table 1. Hemidiaphragms ground either in picric acid solution or Christensen's medium give values for glycine and αamino nitrogen content that are slightly higher than those

of control unground hemidiaphragms which have been simply stored in picric acid solution overnight (approximately 15 hours) at 4°C. Though the small additional yield using the grinding procedure is probably significant, its biochemical validity is questionable since the increment following grinding may well be artefactual. Furthermore, no effect of grinding is detectable in diaphragms previously incubated with GG to provide high glycine levels. For these reasons, the grinding technique has been abandoned in favor of the simple immersion of hemidiaphragms in picric acid solution in the cold overnight.

After overnight storage at 4° C the tubes containing both control and incubated hemidiaphragms suspended in picric acid solution are centrifuged for 15 minutes at 1800 r.p.m. and the supernatants, together with those obtained from similarly treated media, are subjected to analysis for glycine content (Schwartz, Robertson and Holmes, 1955). One milliliter aliquots of picric acid supernatants are selected for the measure-

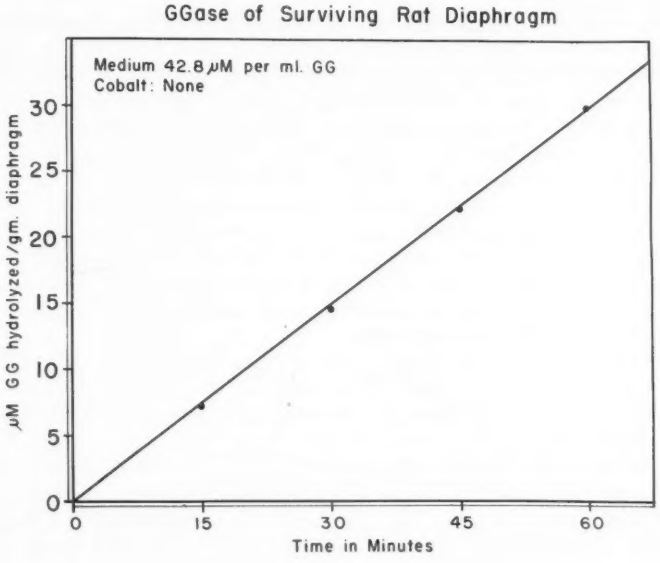

FIG. 1

ment of blanks and standards as well as for control hemidiaphragm determinations, while 0.5 ml. is usually required for determinations of glycine concentration of incubated hemidiaphragms and media.

Calculations.—The GG hydrolyzed, expressed as μM per gram of diaphragm is determined as $\frac{1}{2}$ $(GD_i+GM_i-GD_0)$ where GD_i and GD_0 represent the free glycine concentration in the incubated and control hemidiaphragms, respectively, and GM_i represents the glycine concentration of the incubated medium. All values, including that of the incubated medium, are expressed as μM of glycine per gram of diaphragm.

Estimation of GGase Activity.—In view of the multiple, though minor, changes in technique and calculations from those previously described (Schwartz, *et al.*, 1952), the kinetics of the hydrolysis of GG by surviving diaphragm has been re-explored and, as shown in Figure 1, appears to be that of a zero order reaction, as is the case with aqueous extracts of rat muscle (Smith, 1948a). In the results to be reported, the degree of hy-

drolysis of GG at only one time interval (usually 30 minutes) was measured and GGase activity, K, is defined as the *μM GG hydrolyzed per gram of diaphragm per hour.*

Variation in Control Values.—It became obvious early in the course of this study that there was considerable variability from one experiment to another in dipeptidase activity of "normal" surviving diaphragm. Differences in season and in age of the animals probably are partial determinants of control values for dipeptidase activity but the evaluation of these factors was greatly hampered by recurrent nondescript pulmonary infections which plagued the rat colony and which probably accounted, in large part, for the observed shifts in control determinations of dipeptidase activity.

Fortunately, the spread of values for any *single* experiment was relatively small. This fact permitted completion of the study since, in all experiments, procedures were carried out concurrently, alternating control and experimental animals, all of similar weights. Obviously infected animals were discarded. These precautions, while greatly increasing the requisite number of control animals, provided clear-cut results and minimized hazards in interpretation.

Adrenalectomy.—Animals were transferred from the rat colony to an aid-conditioned room where bilateral adrenalectomy, using a posterior approach, was performed on one-half of the group. A single injection of 0.1 ml., containing 0.5 mg., of desoxycorticosterone acetate in oil was given subcutaneously in the immediate postoperative period. All animals were fed the Purina chow stock diet *ad lib.* and maintained on water containing 1% sodium chloride. Estimation of dipeptidase activity of diaphragms of control and adrenalectomized animals was carried out on the tenth postoperative day.

Exposure to Adrenal Steroids in vitro.—The experimental procedure, as described above, was carried out unchanged, except that all (including "wash") media of the experimental group contained 0.1 mg. of cortisone (Table 3, No. 1). When hydrocortisone was added *in vitro*, again the same procedure was followed except that the washing period was extended to 1 hour, in both control and experimental groups, to permit more prolonged contact of steroid and diaphragm.

Administration of Cortisone Acetate.—All animals treated with cortisone received 5 mg. of a microcrystalline suspension of cortisone acetate (Merck) contained in 0.2 ml. volume and injected intraperitoneally. Injections were given daily for 5 days prior to and including the day GGase activity was determined.

Addition in vitro of Cobalt or Glutathione.—In the experiment in which cobalt (Table 3, No. 5) was added *in vitro*, all media, for both experimental and control groups, were made 0.001 *M* with respect to cobalt sulfate. Similarly, all media were made 0.03 *M* with respect to glutathione in the experiment summarized in Table 3, No. 6.

RESULTS

Effect of Adrenalectomy.—The upper half of Table 2 is representative of values obtained in normal animals. In general, the glycine content of normal rat diaphragm is somewhat lower than previously reported (Schwartz, *et al.*, 1952) because, as discussed above, the maceration of the hemidiaphragm was omitted from the analytical procedure.

As indicated in Table 2, dipeptidase activity (K) of surviving diaphragm is sharply reduced following adrenalectomy. This response results largely from a reduction in the glycine content of the incubating medium, an observation of limited significance which should not be construed as an indication of extracellular hydrolysis. Previous studies (Schwartz, *et al.*, 1952) provided ample evidence that enzymic hydrolysis of glycylglycine

occurs almost entirely *within* the suspended hemidiaphragm. The glycine content of the incubated medium serves only as a repository for glycine which has emerged from the diaphragm following cleavage of the dipeptide. Since, in our experience, the incubated diaphragm is incapable of concentrating free glycine much above $5\mu M$ per gram regardless of how high the glycine content of the incubated medium rises, changes from control values are more likely to be reflected in the incubated medium.

Although Friedberg and Greenberg (1947) observed a significant increase in the total free amino acid content of skeletal muscles of adrenalectomized animals, this change is not demonstrable to any significant degree when free glycine content of rat diaphragm is measured.

Exposure to Adrenal Steroids in vitro.—Hemidiaphragms were suspended

TABLE 2. EFFECT OF ADRENALECTOMY ON DIPEPTIDASE ACTIVITY OF SURVIVING RAT DIAPHRAGM

	Glycine content			K*
	Control hemi-diaphragm	Incubated hemi-diaphragm	Incubated medium	
	μM per gm. diaphragm			
Normal animals (8)	1.3 ±0.30†	4.8 ±0.20	15.6 ±0.69	19.5 ±0.74
Adrenalectomized animals (5)	1.4 ±0.11	4.4 ±0.12	11.1 ±0.58	14.0 ±0.71
P	>0.5	<0.1	<0.01	<0.01

* K = M of GG hydrolyzed per hour per gm. diaphragm.
† Mean ±Standard Error.
Numbers in parentheses represent the number of animals used.

in media containing 0.1 mg. of free cortisone or hydrocortisone for periods of 1 and 1½ hours, respectively. As indicated in Table 3, Nos. 1 and 2, no change in dipeptidase activity from control values is discernible under the conditions of these experiments.

Cortisone Administration.—In contrast, when 5 mg. of cortisone acetate per day are injected intraperitoneally over a 5 day period (Table 3, No. 3) impressive increases of diaphragm GGase activity are repeatedly evident despite the variability from one experiment to another in control values. It was conceivable that these results could be specious in the sense that the increased free glycine concentration from which dipeptidase activity is calculated, might have arisen from steroid-induced increased proteolysis rather than from an increased rate of splitting of glycylglycine. That this is not the case is illustrated in Table 3, No. 4. Here, cortisone-treated animals are compared with normal controls in the absence of substrate. Under these circumstances, K becomes a measure only of endogenously produced glycine. It is clear that negligible quantities of glycine are released and that the effect of cortisone administration is inappreciable.

Addition in vitro of Cobalt and Glutathione.—Smith (1948a), showed that GGase activity was strikingly activated in the presence of Co^{++} and similarly inhibited by cysteine and it has been shown (Schwartz, 1953) that these relationships are retained when suriviving rat diaphragm is used as the enzyme preparation.

It became of interest to determine whether cortisone-induced activation would persist in the face of activation by cobalt or inhibition by glutathione which acts in a manner similar to cysteine (Schwartz, 1953). The data recorded in Table 3, No. 5, indicate that when cobalt sulfate is added to the suspending media, diaphragm dipeptidase activity is greatly en-

TABLE 3. INFLUENCE OF ADRENAL HORMONE ON DIPETIDASE ACTIVITY OF SURVIVING RAT DIAPHRAGM

No.	Effect of	K*		P
		Control animals	Experimental animals	
1	Cortisone *in vitro*	21.8 ± 1.70 (3)	19.9 ± 0.35 (4)	>0.2
2	Hydrocortisone *in vitro*	12.1 ± 1.69 (3)	13.4 ± 1.73 (3)	>0.5
3 a	Cortisone acetate *in vivo*	7.9 ± 0.72 (3)	14.6 ± 1.68 (3)	<0.02
b		10.9 ± 0.57 (3)	15.3 ± 1.30 (3)	<0.05
c		16.8 ± 0.65 (4)	24.3 ± 1.24 (4)	<0.01
4	Cortisone acetate *in vivo* with the absence of substrate (GG)	−0.3 ± 0.22 (4)	−0.2 ± 0.32 (4)	>0.5
5	Cortisone acetate *in vivo* with *in vitro* cobalt activation	40.3 ± 2.00 (5)	48.1 ± 1.70 (6)	<0.02
6	Cortisone acetate *in vivo* with *in vitro* glutathione inhibition	0.4 ± 0.09 (3)	0.8 ± 0.08 (3)	<0.05

Numbers in parentheses represent the number of animals used.
* μM GG hydrolyzed per gm. diaphragm per hour.

hanced. Further, the increment remains significantly greater in the cortisone-treated rats than in untreated control animals. Conversely, despite a dramatic inhibition of GGase activity provoked by exposure *in vitro* to gluthathione, the influence of cortisone administration is not obliterated, since treated animals again showed significantly higher dipeptidase activity than that of the untreated control rats.

DISCUSSION

The results of the present study clearly indicate that intracellular dipeptidase activity of surviving rat diaphragm is diminished following adrenalectomy and increased after cortisone administration. If a primary hydrolytic role of the enzyme under these conditions is accepted, these findings are quite compatible with the thesis that the negative nitrogen balance, which, initially at least, follows cortisone administration, results from an enhanced activity of intracellular proteolytic enzymes. This is

not to say that cortisone has no inhibitory influence on protein anabolism since there is evidence (Hoberman, 1950) that both influences are manifested concurrently.

The cortisone-induced acceleration of peptidase activity of a muscle preparation composed for the most part of *intact cells* contrasts sharply with previous unsuccessful efforts (Engel and Schwartz, 1951) to achieve the same result with *extracts* and *cell-free suspensions* of a variety of tissues subjected to hormonal stimulation. These observations are compatible with the growing conviction among some investigators that hormones, generally, act at intact cell surfaces. However, pertinent exceptions to such an hypothesis have been documented. Kochakian and Robertson (1951) noted increased arginase activity of kidney homogenate following cortisone acetate pellet implantation. Umbreit and Tonhazy (1951a) found that the decrease in proline oxidase activity of kidney homogenate from adrenalectomized rats could be prevented by cortisone administration. Both Cagen *et al.* (1950) and Umbreit and Tonhazy (1951b) have observed accelerated D-amino oxidase activity of rat liver homogenates following the administration of adrenal steroids.

Exposure of rat diaphragm to cortisone and hydrocortisone *in vitro* provoked no response in GGase activity, a finding that parallels the experience of Umbreit and Tonhazy (1951c). Umbreit (1951a) suggested, on the basis of such observations, that cortisone exerts its influence by promoting the formation of additional enzyme protein rather than by activating enzyme already present. Our data dealing with cobalt activation and glutathione inhibition are not inconsistent with this view but allow for other interpretations as well. The stimulating effect of cortisone administration on rat diaphragm dipeptidase activity remains clearly apparent in the face of striking activation *in vitro* by cobalt or equally impressive inhibition by glutathione. If cortisone exerted its action by increasing available activator or by effective removal of an inhibitor, it would be expected that the relatively modest hormonal effect would be masked by the maximal responses provoked by optimal quantities of activator or inhibitor present in the incubating medium. Since cortisone "activation" is not abolished by these measures these experiments provide indirect evidence against the notion that mediation of the hormonal effect occurs by way of physiologic changes in the availability of activator or inhibitor.

How, then, does cortisone exert its influence on intracellular dipeptidase activity? The solution to this problem remains obscure and theoretical possibilities still include (1) stimulation of the cell to produce an absolute increase in enzyme protein, and (2) variation in the internal environment of the cell to provide more optimal conditions for enzyme activity. Appropriate changes in pH or increased exposure to substrate are conceivable examples of the second possibility. It will be of interest to determine whether dipeptidase will exhibit the phenomenon of adaptation in a manner

similar to tryptophane oxidase (Knox and Mehler, 1951) and, if so, whether this form of "activation" is distinct from the accelerating effect of adrenal steroids.

SUMMARY

By the use of a procedure which permits quantitative measurement of the rate of cleavage of glycylglycine (dipeptidase activity) by surviving rat diaphragm the following observations were made:

1. Dipeptidase activity was significantly lowered following adrenalectomy.

2. Cortisone administration to rats resulted in an appreciable increase in dipeptidase activity. This stimulating effect of adrenal steroids was not demonstrable *in vitro*.

3. The increase in dipeptidase activity due to cortisone administration persisted even though dipeptidase activity is strongly activated by cobalt or inhibited by glutathione.

Some of the implications of these findings with regard to the nature of protein catabolic effect of corticosteroids are discussed.

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THE EFFECT OF HYPOPHYSECTOMY, REMOVAL OF THE POSTERIOR PITUITARY, ADRENALECTOMY, AND DEHYDRATION ON THE ANTIDIURETIC ACTIVITY OF THE RAT HYPOTHALAMIC-NEUROHYPOPHYSEAL SYSTEM

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THE many investigations on the hypothalamic-neurohypophyseal system which have been reported in recent years (see the reviews by Scharrer and Scharrer, 1954 and Stutinsky, 1953) have brought forth new concepts as well as contradictory statements. With the aid of Gomori's chrome-hematoxylin phloxin stain the role of the neurosecretory material formed in the supraoptic nuclei and paraventricular nuclei of the hypothalamus has been more clearly defined. Experimental evidence supports the view that the neurosecretory substance contains the active principles of the posterior pituitary. However, there are still a number of problems, particularly with respect to the role played by the different parts of the hypothalamic-neurohypophyseal system in the control of water metabolism. As a contribution toward the solution of some of these problems, a number of experiments were undertaken designed to assess the antidiuretic activity of different components of the hypothalamic-hypophyseal system under various conditions such as dehydration, and following surgical procedures, such as total hypophysectomy, removal of the posterior pituitary alone and adrenalectomy.

METHODS AND MATERIALS

Male rats (approx. 60 days of age) of the Long Evans strain were used. Most of the animals, with the exception of the following groups, received the regular laboratory diet prepared in the Institute of Experimental Biology of the University of California. The adrenalectomized animals maintained on 1% NaCl and the hypophysectomized animals were given a special high protein diet. A NaCl-free diet was offered to one group of adrenalectomized rats.

The animals which were hydrated to serve for the bioassay of the antidiuretic material will be referred to as *recipients*, and the animals whose tissues were assayed will be designated as *donors*.

Birnie's method (1950) for assaying the antidiuretic activity was employed in order to establish a high diuretic rate. After hydration of the recipients, the first urine collection was made. Then the abdomen was shaved, and an incision was made intraperitoneally with a 16 gauge needle. After the needle was withdrawn, a cannula was directed

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through the opening, and the tissue to be assayed was washed into the peritoneal cavity with 2 cc. of 0.9% saline.

The urine collections were recorded at 60 minutes, 90 minutes, 4 hours, and 8 hours. These data were used to calculate what per cent of the water present at the time of injection of the tissues had been excreted by the animal at the designated intervals. The per cent dose excreted at 90 minutes after the injection of the test material was adopted as the significant interval, for by this time the hydrated non-injected animal had excreted approximately 70% of the water. The observations were continued until 8 hours had passed, because the 90 minute reading did not represent the total duration of antidiuretic activity.

In one group of recipients the posterior lobe of the pituitary alone was removed. These animals were observed until diabetes insipidus had ensued and then were hydrated in a manner identical to that of the normal recipients.

TABLE 1. DIVISION AND TREATMENT OF ANIMALS AND RESULTS

Experimental conditions	No. of donors	Tissue assayed	% water dose excreted by recipients in 90 min.
I. Normal controls: (untreated animals)	6	Neurohypophysis	12% (7–21)
	6	Supraoptic area	17% (9–24)
	6	Median eminence and stalk	52% (22*–71)
	6	Posterior lobe	15% (7–38)
II. Saline controls: (2 cc. 0.9% NaCl injected intraperitoneally)	0	8 recipients	67% (62–81)
III. Hypophysectomy: (34 days postoperative)	8	Supraoptic area	51% (33–75)
	14	Median eminence and stalk)	71% (40–84)
IV. Dehydration:			
A. 5% NaCl, 4 ml/100 gms. body weight. Injected subcutaneously			
1. sacrificed after 5 hours	6	Supraoptic area	14% (2–23)
	6	Neurohypophysis	12% (4–17)
2. sacrificed after 12 hours	6	Supraoptic area	23% (14–39)
	6	Neurohypophysis	34% (12–64)
B. No drinking water for nine days	8	Supraoptic area	27% (9–47)
	8	Neurohypophysis	43% (36–59)
V. Adrenalectomy: (ether anesthesia)			
A. 7 days post operative with 1% NaCl	7	Supraoptic area	16% (9–30)
	7	Neurohypophysis	14% (10–24)
B. 7 days post operative with NaCl free diet and distilled drinking water	7	Supraoptic area	21% (12–34)
	7	Neurohypophysis	17% (7–23)
VI. Removal of posterior pituitary from donors: (19 days post-operative)	5	Supraoptic area	46% (34–64)
	5	Median eminence and stalk	69% (53–79)
VII. Recipients with no posterior pituitary hydrated for assay of normal donor tissue:	6	Supraoptic area	11% (2–15)
	5	Neurohypophysis	9% (2–19)

* This animals caught up with the excretion rate of the other animals at the next reading.

After two other techniques had been tried, the following method was employed for the preparation of the hypothalamic and neurohypophyseal tissues for assay. After decapitating the unanaesthetized animals and removing the brain, the tuber cinereum with the median eminence and stalk was excised with a razor blade and set aside temporarily to be added to the posterior pituitary to complete the neurohypophyseal tissue. From histological sections previously prepared, the limits of the supraoptic nuclei were defined so that a block of tissue was severed, beginning rostral to the optic chiasm, extending caudally to the interpeduncular fossa, laterally along the optic tracts to the approximate position of the lateral geniculate bodies and dorsally in a plane passing through the anterior commissure. The block of tissue was comparable to that prepared by Hare (1937) from dogs and was referred to as the supraoptic area. The separated pieces, the supraoptic area and the neurohypophysis, were placed in separate glass cannulas and injected into the recipients' peritoneal cavities. The groups employed are indicated in Table 1.

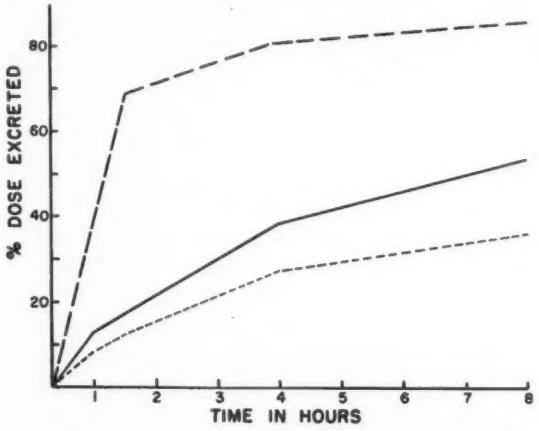

FIG. 1. The average per cent of the total water dose excreted during an eight hour period by normal animals injected with normal supraoptic tissues (———), neurohypophyses (– – –), and 0.9% saline (— — —).

RESULTS AND DISCUSSION

The data from these experiments are represented by linear graphs which indicate the average per cent dose excreted over an eight hour test period. Figure 1 shows that a greater quantity of urine is excreted consistently by those animals receiving the hypothalamic tissue than by those receiving the neurohypophysis, signifying that the concentration of antidiuretic substance in the supraoptic area is less than in the neurohypophysis. At the 4-hour period the difference is evident: i.e. 38% (22–56) of the dose is excreted by those animals receiving the supraoptic area in contrast to 24% (8–29) excreted by those injected with the neurophypophysis. The saline recipients showed no apparent antidiuretic effect. Fig. 1.

Figure 2 displays the marked difference in concentration of the antidiuretic substance in the parts of the neurohypophysis throughout the eight hour observation period.

As mentioned in the beginning, the hypothalamus and neurohypophyseal system both have substances in common, namely, an antidiuretic substance and neurosecretory material. It is reasonable to assume that these two substances are closely related, but there is no absolute proof that the neurosecretory material is the antidiuretic substance or *vice versa*. For example, in the hypothalamus of the rat little stainable material can be demonstrated with the aid of the chrome alum hematoxylin stain (Ortmann 1950, Diamond 1952), yet a considerable amount of the antidiuretic substance is present. In the posterior pituitary of the rat, however, both neurosecretory material and the antidiuretic substance are found in large quanti-

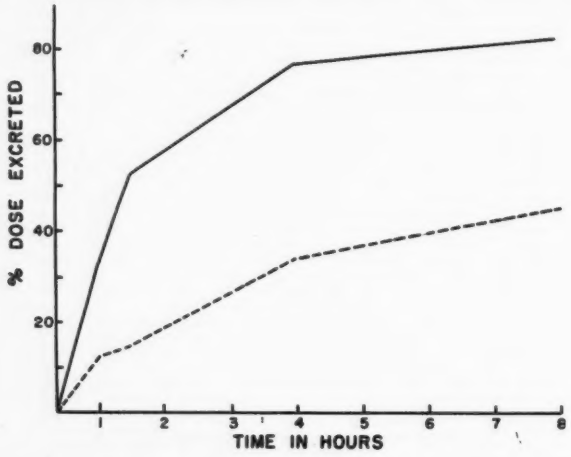

FIG. 2. The average per cent of the total water dose excreted by normal hydrated animals injected with posterior lobes (– – –) and with the combined median eminence and stalk (———) from normal animals.

ties. In addition, it has been pointed out that with Halmi's modification of the aldehyde fuchsin stain of Gomori, the neurosecretory material in the rat hypothalamus does stain and is present in every cell in the supraoptic nucleus (Scharrer, personal communication). The question is raised then why one stain demonstrates the granules in the hypothalamus and the other does not, whereas both show the neurosecretory material in the posterior pituitary? Possibly the answer lies in the size of granules and that one of the stains is not critically absorbed by the small granules or, perhaps, the hypothalamic neurosecretory substance is somewhat modified before reaching the posterior pituitary, that is, the large parent molecule is changed but not the active antidiuretic component.

Furthermore, as is well known, neurosecretory material is not limited to the vertebrate hypothalamic neurohypophyseal system alone, but is also found in invertebrate structures, some of which have not been proven to be associated with a water balance mechanism or, more precisely, with an

antidiuretic substance. However, some experimental evidence has accumulated to show that in certain instances the two substances, antidiuretic substance and neurosecretory material, do show similar reactions, and, thus, allow one to make comparisons between their possible relationships.

Hypophysectomy and Stalk Section:

Many investigators have demonstrated the accumulation of nuerosecretory substance on the proximal side of a cut stalk (Stutinsky 1951, Hild 1951, Scharrer and Wittenstein 1952, Billenstien and Leveque 1955). Scharrer and Wittenstein reported that "soon after" stalk section in dogs the neurosecretory substance accumulated proximal to the cut, but in animals killed "several weeks" following the operation the neurosecretory material had ceased to accumulate. If an experiment involving complete hypophysectomy in rats can be compared with stalk section in dogs, and if one can compare the antidiuretic substance with the neurosecretory material, the present data support Scharrer's and Wittenstein's work in that thirty four days after hypophysectomy no increase in the antidiuretic substance is found in the stalk and median eminence (Fig. 3).

Several authors (Stutinsky 1951, Scharrer and Wittenstein 1952, and Billenstien and Leveque 1955) have studied the reorganization of the cut pituitary stalk which, after some months following hypophysectomy, assumes the histological appearance of a normal neural lobe. With the method employed an actual decrease in the amount of antidiuretic substance was detected in the remaining portion of the stalk after hypophysectomy in contrast to the positive findings of Sato (1928) and Lloyd and Pierog (1955) (Fig. 3). It does seem peculiar that the hypothalamus should contain more antidiuretic substance thirty days after hypophysectomy than it does recently after the operation, as reported by the latter authors, for many investigators have shown that the hypothalamic cells, which are supposedly producing the antidiuretic substance, display various degrees of degeneration after hypophysectomy or stalk section (Fisher 1938, Magoun and Ranson 1939, Rasmussen 1940, Rasmussen and Gardner 1940, Heinbecker and White 1941, Frykman 1942, Keller 1942, O'Connor 1947–1948, Bodian and Maren 1951, Diamond 1952, and Vogt 1954). The reason for these discrepancies may be that since assays of hypothalami after hypophysectomy refer to blocks of hypothalamic tissue with or without the reorganized remnant of the stalk after different periods of reorganization, the different investigators obtain different results.

On the other hand, if the reorganized tissue is a newly formed posterior lobe, one would expect it to become depleted of the neurosecretory material in a manner comparable to that of the neural lobe after the customary methods of dehydration (shown by Ortmann 1950, 1951, Hild 1951, Hild and Zetler 1953b, Leveque and Scharrer 1953). However, Billenstien and Leveque (1955) have shown that this accumulated material did not fluctuate after dehydration but only with an injection of desoxycortico-

sterone acetate did the dehydrated animal become depleted of the material. Yet Lloyd and Pierog (1955) showed a decrease in the antidiuretic substance after dehydration under what seem to be conditions comparable to those employed by Billenstien and Leveque, pointing out the fact that in not all cases do the antidiuretic substance and neurosecretory material react similarly to external stimuli. It appears that the logical conclusion to draw from the inconsistencies mentioned is that one must consider the precise region of stalk section in making comparative studies, a suggestion pointed out by Fisher in 1938 and again by Billenstien and Leveque in 1955.

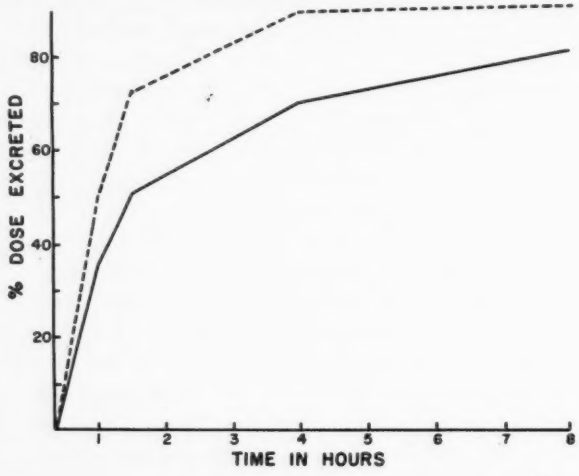

FIG. 3. The average per cent of the total water dose excreted by normal hydrated animals injected with the supraoptic tissue (———) and the combined median eminence and stalk (– – –) from animals hypophysectomized for thirty four days.

Posterior Lobectomy:

Contrary to the suggestion of Gaupp and Scharrer (1935), Heinbecker and White (1941) believed that after posterior lobectomy the supraoptic nucleus does not secrete any antidiuretic substance. The present data support the latter group of investigators, for the amount of antidiuretic substance left in the supraoptic area nineteen days after posterior lobectomy is decreased in an amount comparable with that after complete hypophysectomy. Also the fact that dehydration by water deprivation shortly after hypophysectomy did not produce any increase in the antidiuretic substance in the urine would tend to support the findings that no antidiuretic substance is being released from the remainder of the neurohypophysis (Gilman and Goodman 1937).

The question has been raised as to whether the antidiuretic substance produced by the hypothalamus is in some way modified or activated by the pituitary before being released into the blood stream. The present study shows that the hypothalamic material is potent in the absence of any pos-

terior pituitary (Fig. 6). Thus, there appears to be no modification of this substance by the pituitary, as is suggested by Vogt (1954) in regard to the oxytocic activity. The physiological release of the antidiuretic substance from the intact neurohypophyseal system may be dependent, however, upon some other factor such as the pituicytes of the posterior pituitary, as suggested by Hild (1951).

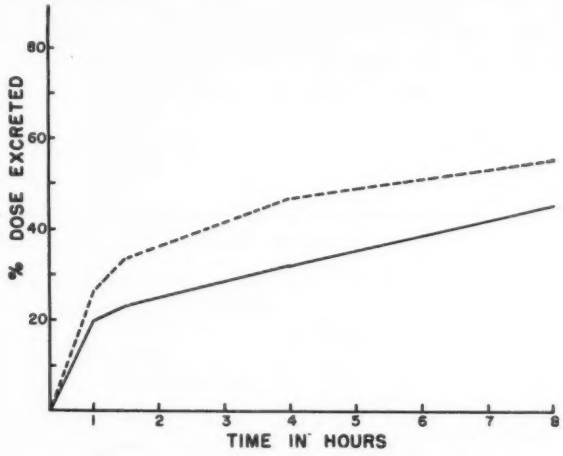

FIG. 4. The average per cent of the total water dose excreted by normal, hydrated animals injected with supraoptic tissues (———) and the neurohypophyses (– – –) from animals which received 5% NaCl and were sacrificed after 12 hours.

Dehydration:

According to Kovács and Bachrach (1951) the intraperitoneal administration of 5% NaCl solution reduced the antidiuretic activity after one hour in both the hypothalamus and hypophysis. In the present study using half as much saline and a subcutaneous mode of administration, no alteration in the concentration of the antidiuretic substance was noted in either tissue following a four hour period. After twelve hours, however, the antidiuretic substance had decreased in the posterior lobe (Fig. 4); this is in agreement with the work of Chambers (1945), who reported that the posterior pituitaries of rats showed a 40% loss in antidiuretic activity twelve hours after the injection of 3–6 cc. of a 5% NaCl solution into the tail vein.

Another method of dehydration, water deprivation, gave results similar to those obtained by the salt injections. After nine days of dehydration the antidiuretic activity in the hypothalamus had not significantly changed, whereas that of the pituitary had decreased (Fig. 5). These results uphold the histological findings of Ortmann (1950), who demonstrated that after dehydration the neurosecretory substance had completely disappeared from the pituitary, whereas granules in the hypothalamus remained within normal variation.

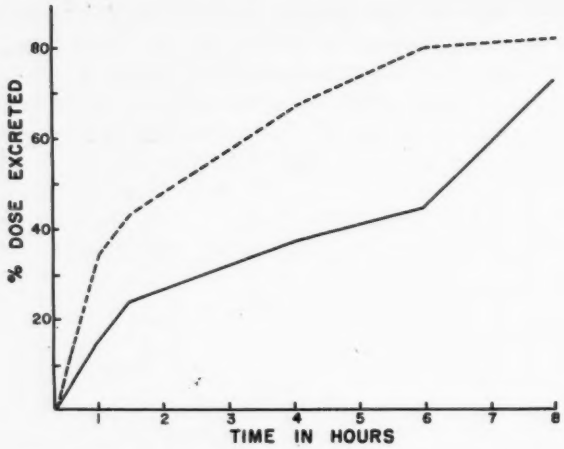

FIG. 5. The average per cent of the total water dose excreted by normal, hydrated animals injected with supraoptic tissues (———) and neurohypophyses (– – –) from animals which were dehydrated for nine days.

Adrenalectomy:

In the experiments of Kovács and Bachrach (1951) and Cavellaro (1954), adrenalectomy caused a quantitative decrease in the antidiuretic substance of the neurohypophyseal hypothalamic system. Eichner (1953) and Mirsky *et al.* (1954) found an increase in the antidiuretic substance in the blood after adrenalectomy. On the other hand, Gersh and Grollman

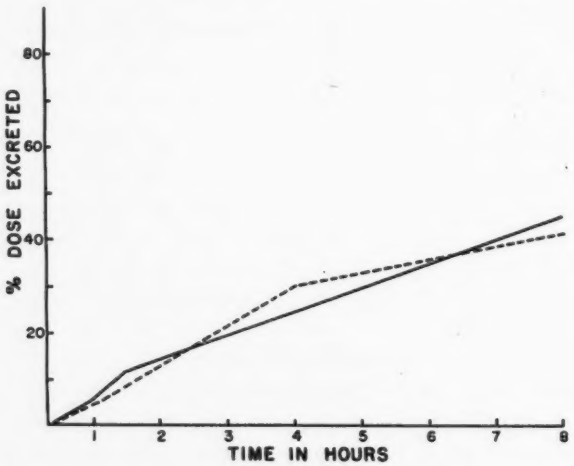

FIG. 6. The average per cent of the total water dose excreted during eight hours by hydrated animals injected with normal supraoptic areas (———) and neurohypophyses (– – –). These hydrated animals had previously had their posterior pituitaries removed.

(1939) and Ames and Van Dyke (1952) concluded, respectively, that there was no evidence of cellular stimulation in the posterior pituitary after adrenalectomy and that there was no increase in the amount of antidiuretic substance in the blood following removal of the adrenals. In the present study, no change was noted in the concentration of the antidiuretic substance in the supraoptic hypophyseal system seven days after severe adrenal insufficiency. The results tend to support the work of Birnie *et al.* (1950, 1953) that an increase in the antidiuretic substance in the urine three to seven days after adrenalectomy may be accounted for by the decreased destruction in the liver rather than an increased release from the posterior pituitary.

Admittedly, the present methods of assay were not as discriminating as were those reported in the paper by Cavallero *et al.* (1954), but in most cases these methods proved adequate to detect large changes in the antidiuretic substance of the assayed tissues. It is not intended to pursue the critical evaluation of the results of other investigators, therefore, much farther. Evidently, different results may well be explained by differences in techniques.

SUMMARY

The antidiuretic activity was measured in the hypothalamic-neurohypophyseal system of male rats (approx. 200 gm.) after subjecting the animals to various experimental conditions, such as dehydration, both by saline injection and by water deprivation, adrenalectomy, complete hypophysectomy, and removal of the posterior lobe of the pituitary.

Of the various conditions employed, those involving dehydration were the only ones to cause a marked change in the concentration of the antidiuretic substance in the neurohypophysis. The fact that the concentration of the antidiuretic substance in the supraoptic area remained unchanged under these same conditions would seem to indicate that the antidiuretic substance was being formed in the supraoptic area and then stored in and released from the posterior pituitary.

Evidence was obtained that the presence of the posterior lobe of the pituitary as an activator is not necessary for the hypothalamic material to display antidiuretic activity.

A decrease in the quantity of the antidiuretic substance was noted in the hypothalamus and in the remaining portion of the neurohypophysis thirty four days after hypophysectomy and 19 days after removal of the posterior lobe.

The evidence that the antidiuretic substance in the hypothalamus and neurohypophysis is not altered several days after adrenalectomy, and yet has been reported to increase in the circulatory system, supports the suggestion that there may be decreased destruction of the substance by the liver rather than increased production by the hypothalamic-hypophyseal system.

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THE BIOLOGICAL ACTIVITY OF A NEW DCA DERIVATIVE $\Delta^{1,4}$-PREGNADIENE-21-OL-3,20-DIONE, 3-ACETATE

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MARKED qualitative and quantitative alterations in biological activity often follow minor changes in the structural features of the steroid hormones. This fact has been cogently demonstrated by the recent introduction of two new synthetic steroids, $\Delta^{1,4}$-pregnadiene-17α,21-diol-3,11,20-trione (Δ^1-Cortisone) and $\Delta^{1,4}$-pregnadiene-11β,17α,21-triol-3,20-dione (Δ^1-Cortisol). The chemistry and clinical effectiveness of these derivatives of Cortisone and Cortisol have been reported (Herzog, *et al.*, 1955; Bunim, Pechet and Bollet, 1955; Gray and Merrick, 1955). $\Delta^{1,4}$-Pregnadiene-21-ol-3,20-dione, 3-acetate (hereafter referred to as Δ^1-DCA) was synthesized in our laboratories by Clarke *et al.* (1955). This new steroid differs chemically from DCA (Δ^4-Pregnene-21-ol-3,20-dione, 3-acetate) in that it possesses a double bond in the 1:2 position of ring A of the steroid nucleus as do Δ^1-Cortisone and Δ^1-Cortisol.

The purpose of this report is to describe certain of the biological activities of Δ^1-DCA which are compared with DCA, its nearest relative, in the following tests: 1) life maintenance in adrenalectomized rats 2) interference with estrogen-induced alteration in pituitary function and 3) inhibition of histamine-induced ACTH discharge by the pituitary. Studies comparing Δ^1-DCA and DCA as to their relative effects on salt and water metabolism and on cardiovascular phenomena are being reported separately by Nairn, Masson and Corcoran (1955).

METHODS AND MATERIALS

Life maintenance test. This test was conducted essentially in accordance with the assay method described by Cartland and Kuizenga (1936). Δ^1-DCA and DCA dissolved in sesame oil were injected subcutaneously, daily for 20 consecutive days, into bilaterally adrenalectomized female rats. A record was kept of the number of days each animal survived within each experimental group of 8 to 10 rats. Animals which survived an additional 20 days after the last injection were necropsied. The para-renal tissue was tested with silver nitrate (Szent-Györgi, 1928) for the presence of accessory adrenal tissue. Five rats which exhibited a positive silver nitrate reaction were discarded from the test.

Effects on estrogen-induced alteration in pituitary function. Young male rats weighing 160 to 180 gm. were employed. To each experimental animal 0.114 mg./kg. of diethylstilbestrol dipalmitate (DESDP) was injected subcutaneously in cottonseed oil, daily

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except Sunday, for 2 weeks. This dose of estrogen was chosen after previous studies had shown that this amount was sufficient to bring about a statistically significant ($P = <0.01$) increase in the weight of the adrenals and a similarly significant decrease in the weight of the seminal vesicle and ventral prostate glands of young mature rats.

Δ^1-DCA and DCA, as suspensions in cottonseed oil, were each given at two dose levels together with the estrogen. The injections were made subcutaneously at different sites. Control groups consisted of rats which received the vehicle only.

Approximately 24 hours after the last injection the rats were sacrificed under chloroform. The adrenals, pituitary, testes, ventral prostate and seminal vesicles were excised, cleaned, blotted and weighed on an appropriate balance.

Inhibition of the histamine-induced release of ACTH. The procedures used for this study were mainly those described by Hodges (1953) except that histamine diphosphate was used as the stressor agent instead of operative trauma. Twenty-two hours after graded doses of Δ^1-DCA or DCA, suspended in cottonseed oil, had been injected subcutaneously in male Sprague-Dawley rats, a standard dose (20 mg./kg. of rat) of the histamine base was injected subcutaneously. One hour after the administration of the amine, the experimental animals were sacrificed under sodium pentabarbital anesthesia. The adrenal glands were removed, cleaned and weighed. The content of ascorbic acid in the adrenals was determined by the method of Roe and Kuether (1943).

RESULTS

Life maintenance. The results of this test are presented in Table 1. Mean survival times show that Δ^1-DCA is capable of extending the life of adrenalectomized rats to a degree proportional to dose. Analysis of variance of

TABLE 1. THE COMPARATIVE LIFE MAINTENANCE ACTIVITY OF Δ^1-DCA AND DCA IN YOUNG ADRENALECTOMIZED RATS

Treatment	Dose: mg./rat/day	No. of rats	Ave. body weight (gm.)		No. surviving 20 days	Average survival time (days)
			Initial	at 20 days		
Controls	0	10	49	—	0	5.6
DCA	0.0125	10	51	102	2	11.0
	0.025	8	47	101	4	14.8
	0.5	9	47	101	2	12.2
	0.1	10	49	111	7	16.4
Δ^1-DCA	0.025	10	52	—	0	6.8
	0.05	9	51	—	0	9.6
	0.1	10	53	102	3	12.3
	0.2	9	52	115	5	15.1

the data (method of Bliss and Marks (1939)) indicated that Δ^1-DCA was $\frac{1}{4}$ as active as DCA in this respect.

Estrogen-induced alteration in pituitary function. The ability of natural and synthetic estrogens to inhibit the elaboration of pituitary gonadotrophic hormones (*cf.:* review article by Greep and Jones, 1952) and to stimulate the release of ACTH (Ingle, 1936; del Castillo and Sammartino, 1937; and Gemzel, 1952) has been well established. The ability of Δ^1-DCA to modify estrogen-induced pituitary changes has been compared with that of DCA (Table 2).

The dose of DESDP employed (0.114 mg./kg./day for 14 days) produced a statistically significant increase ($P < 0.01$) in the pituitary and adrenal weights as well as a highly significant decrease ($P = 0.01$) in the weight of the seminal vesicle and ventral prostate glands. This dose of estrogen, however, was inadequate to modify the growth rate of the animals or to produce testicular atrophy.

DCA at 20 mg./kg./day partially prevented the DESDP-induced adrenal weight increase. On the other hand, Δ^1-DCA at the same dose level completely prevented the adrenal hypertrophy brought about by estrogen.

TABLE 2. COMPARATIVE EFFECTS OF Δ^1-DCA AND DCA ON ESTROGEN-INDUCED CHANGES OF ENDOCRINE ORGANS

Treatment	No. of animals	Dose, mg./kg./day	Mean body weight (gm.)		Organ weights (mean ± s.e.) milligrams				
			Initial	Final	Pituitary	Adrenal	Testes	Seminal vesicles	Ventral prostate
Control	8	—	178	218	7.7 ± 0.33	30.1 ± 1.9	2770 ± 86.0	179 ± 7.9	176 ± 12
DESDP†	6	0.114	171	209	10.5 ± 0.42	45.5 ± 2.6	2870 ± 127	102 ± 20	118 ± 16
DESDP + DCA	3*	0.114 + 5.0	175	218	10.7	41.3	2920	82	95
DESDP + DCA	6	0.114 + 20.0	169	207	10.1 ± 0.39	39.2 ± 1.5	2370 ± 159	66 ± 11	73 ± 7.7
DESDP + Δ^1-DCA	3*	0.114 + 5.0	173	208	10.3	41.1	2490	118	98
DESDP + Δ^1-DCA	6	0.114 + 20.0	173	206	10.1 ± 0.58	28.4 ± 1.9	2700 ± 90	103 ± 16	98 ± 15

* Standard error of the mean not calculated due to small group size.
† Diethylstilbestrol dipalmitate.

From this it is concluded that Δ^1-DCA is considerably more active than DCA in blocking the mechanism in rats which is responsible for the enlargement of the adrenals following massive estrogen administration.

The doubly unsaturated derivative did not modify the suppressive effect of estrogen on the reproductive system of the male rat, whereas DCA with DESDP produced a greater atrophy of the seminal vesicle and ventral prostrate glands than did the estrogen alone. This suggests that Δ^1-DCA does not interfere with the elaboration of pituitary gonadotrophin at dose levels which inhibit ACTH release.

Inhibition of the histamine-induced ACTH discharge. The observed greater effectiveness of Δ^1-DCA compared to DCA in preventing estrogen-induced adrenal hypertrophy suggested that Δ^1-DCA might have a correspondingly greater capacity to block histamine-induced ACTH discharge by the pituitary. To test the possibility, ACTH stimulation, as determined by depletion of adrenal ascorbic acid, was followed (Table 3).

The ascorbic acid content of adrenals from untreated rats was 419 ± 12 mg. %. Histamine diphosphate, 20 mg./kg. given subcutaneously one hour before removal of the adrenals, brought about marked depletion of

the vitamin. In two experiments, adrenal ascorbic acid following histamine was 323 ± 16 and 335 ± 13 mg. %. The effectiveness of Δ^1-DCA and DCA in reversing this histamine reaction was, for all practical purposes, identical. Either compound, at a dose level of 50 mg./kg. 22 hours before autopsy, was capable of blocking almost completely the discharge of ACTH normally induced by histamine. Both steroids, at a dose level of 100 mg./kg., not only completely blocked the histamine effect but also brought about a concentration of adrenal ascorbic acid which was significantly greater ($P < 0.05$) than that of the adrenals of untreated animals.

TABLE 3. INFLUENCE OF Δ^1-DCA AND DCA ON HISTAMINE-INDUCED ADRENAL ASCORBIC ACID DEPLETION

Group	No. of animals	Histamine base, mg./kg.	Steroid, mg./kg	Body weight, gm.	Adrenal weight, mg. mean ±s.e.	Adrenal ascorbic acid, mg. per cent mean ±s.e.	Change mg. per cent
Control	6	—	—	106	28.1 ±2.0	419 ±12	—
DCA	8	2.0	—	106	26.5 ±1.4	323 ±16	—
	8	2.0	25	106	26.4 ±1.0	327 ±21	+ 4
	8	2.0	50	106	25.2 ±1.1	408 ±25	+ 85
	6	2.0	100	108	26.3 ±0.9	474 ±7.4	+151
Δ^1-DCA	8	2.0	—	109	29.9 ±1.0	335 ±13	—
	8	2.0	25	109	28.7 ±1.6	334 ±17	− 1
	8	2.0	50	106	25.2 ±1.1	408 ±25	+ 73
	8	2.0	100	105	26.5 ±1.2	472 ±12	+137

DISCUSSION

From the information which has been collected concerning the biological activity of Δ^1-DCA, it would appear that increasing the degree of unsaturation of ring A of DCA does not disturb its adrenocortical hormone activity to a great extent. However, minor but definite changes in degree of activity have been observed. The data indicate that the life-maintaining activity was the most adversely affected by the addition of the 1:2 double bond to DCA, as shown by the fact that Δ^1-DCA was only $\frac{1}{4}$ as active as DCA in supporting life in the adrenalectomized rat. Wettstein (1940) reported that $\Delta^{4,6}$-Pregnadiene-21-ol-3,20-dione, 21-acetate was inactive in the life maintenance test. From this it can be concluded that additional unsaturation in ring B of DCA is considerably more destructive of life-maintaining activity than is a corresponding chemical change in ring A.

The observation that Δ^1-DCA possessed a significantly greater capacity than DCA to prevent the estrogen-induced adrenal hypertrophy is difficult to reconcile with the finding that Δ^1-DCA and DCA were equipotent in preventing the histamine evoked ACTH release by the pituitary, unless it is assumed that the agent responsible for the enlargement of the adrenal subsequent to estrogen treatment is a qualitatively different form of ACTH than that which brings about a prompt depletion of adrenal ascorbic acid after histamine treatment. The evidence that ACTH is the substance

responsible for these two adrenal responses is highly convincing (*cf.*, Ellison and Burch, 1936; del Castillo and Sammartino, 1937; Ingle, 1941; Levin 1945; Skelton *et al.*, 1949; Gemzell, 1952).

Considerable evidence has been advanced recently to support the claim that the ACTH molecule as secreted by the pituitary might consist of at least two dissociable components having separate and distinct actions on the adrenal; one possessing an adrenal growth promoting effect, the other having the capacity to deplete the vitamin C stores of the adrenal. Thus Stack-Dunne (1953) reported that the proteins of crude corticotrophin can be separated chromatographically into fast-moving and slow-moving components. The fast-moving components were assayed to contain predominantly "adrenal weight activity" (50% of total), whereas the slower moving components possessed adrenal ascorbic acid depleting activity with little adrenal weight activity. Evidence suggesting a multiplicity of adrenocorticotrophic hormones has also been presented by Reinhardt, Geschwind and Li (1951) who used the ratio of activities in a variety of assay procedures for different ACTH preparations to point out the possibility that the various activities of ACTH can be relatively dissociated according to the method of preparation and with altered degree of purity.

The observations described in this report, that Δ^1-DCA was more active than DCA in inhibiting the adrenal weight factor of ACTH and was equally as potent as DCA in blocking the ascorbic acid depleting factor of ACTH, are compatible with the concept that the adrenal weight activity and ascorbic acid depleting activity are due to separate and dissociable components of ACTH.

In our hands the amount of DCA which was necessary to effect complete inhibition of the histamine induced ACTH release was considerably less (50–100 mg./kg.) than that (200 mg./kg.) reported by Hodges (1953) to block completely the stress-induced ACTH release subsequent to operative trauma. Since the experimental procedures in each case are nearly identical, except that histamine was used in the studies reported here, it would appear that the differences in the effectiveness of DCA as reported by the two laboratories could be explained on the basis of the differences in the degree of stress imposed upon the experimental animals. Sayers and Sayers (1947) state that the greater the degree of stress to which the animal is subjected, the greater is the amount of cortical hormone required to inhibit pituitary adrenocorticotrophic activity. Therefore, the above explanation seems plausible, particularly since the depletion of adrenal ascorbic acid one hour after surgical trauma was found to be considerably greater than that seen one hour subsequent to the subcutaneous injections of histamine diphosphate.

SUMMARY

Minor but definite changes in the biological activity of DCA are effected by increasing the degree of unsaturation in ring A. Thus, $\Delta^{1,4}$-Pregnadiene-

21-ol-3,20-dione, 3-acetate (Δ^1-DCA) is $\frac{1}{4}$ as active as DCA in the life-maintenance test, more active in preventing estrogen-induced adrenal hypertrophy, and equally active in preventing the depletion of adrenal ascorbic acid following histamine diphosphate administration. The relative differences in activity, with respect to experimentally induced adrenal changes, are accepted as additional evidence in support of the current concept that ACTH may be dissociated into qualitatively different components having separate and distinct biological acitivities.

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EFFECTS OF NEUROHYPOPHYSECTOMY IN DOMESTIC CHICKENS[1]

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IN THE domestic hen 24–26 hours elapse between the time when an ovum first enters the oviduct and the time the completed egg is expelled from the uterus. During this stay, the egg remains in the uterus or shell gland 18 to 20 hours where it acquires the hard shell. When formation of the hard shell is completed, uterine contractions set in which lead to the eventual expulsion of the egg. Little is known about the factors which determine the length of time the egg is gestated in the uterus and those which determine the moment for its expulsion. It remains to be seen whether these factors are similar or even comparable to those which initiate the birth of the uterine contents in mammals.

Among the factors implicated in the initiation of parturition in mammals are the hormones from the posterior lobe of the pituitary gland. Interference with the posterior lobe has been found to result in abnormal or delayed parturition in some mammals while in others no effects were noticed.

No reports on the effects of ablating the posterior lobe of the hypophysis in birds were found in the literature. Riddle (1921) states that the injection of whole posterior pituitary substance into doves, pigeons and chickens resulted in premature expulsion of eggs from the uterus. Burrows and Byerly (1940, 1942) similarly report that various preparations of the posterior lobe of the pituitary caused premature expulsion of uterine eggs especially if these hormone preparations were given intravenously and if the egg was hard shelled. Preliminary work in this laboratory (unpublished) showed that the degree of effectiveness of posterior pituitary hormones was inversely related to the number of hours the egg had remained in the uterus at the time of hormone injection.

Because of the paucity of information on the role of the posterior lobe of the hypophysis in the reproductive cycle of the domestic chicken, a study was undertaken in which the posterior lobe was removed without interference with the adenohypophysis. Because of the frequency and

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regularity with which domestic hens lay, they are exceptionally good experimental subjects for the study of the role of the posterior lobe in the laying cycle.

METHODS

The birds from which the posterior lobe was to be removed were anesthetized with intravenous Nembutal and were secured on the operating board in such a way as to prevent head movement. An incision was made alongside the trachea and lateral to the glottis exposing the buccal cavity. At a point just anterior to the transverse vein, a small incision was made through the mucosa and it and the underlying muscles were retracted exposing the basi-temporal bone. A piece of this bone measuring about one-half centimeter in diameter was excised. With the aid of a dental drill and a bone pick the cancellous bone was removed exposing the caudal end of the sella turcica. Aided by a microscope, the portion of the bony shell surrounding both the posterior lobe and the caudal end of the anterior lobe of the pituitary was carefully removed. A needle was used to slit the dura mater along the slight depression indicating the junction of the two lobes. By lifting the caudal end of the anterior lobe upward the infundibular stalk was exposed. Using a modified fine scalpel and a suction pipette this stalk was severed and the posterior lobe was removed by suction, care being taken not to disturb the portal vessels of the anterior lobe of the pituitary gland. The incisions were closed and the birds were returned to their cages where they recovered from the operation very rapidly and without special care.

For the measurement of the rate of urine flow, the birds were fastened upon an inclined rack at an angle of about 45 degrees. A plastic tube (70 mm. long and 7.5 mm. thick) with perforated walls and one sealed end was inserted into the cloaca so that a small length of tubing remained protruding from the bird. The urine, as it drained from the ureters, flowed into the perforated tube and hence into a funnel and a graduated cylinder.

EXPERIMENTS AND RESULTS

In two experiments, the effects of extirpation of the posterior pituitary gland on growth, feed consumption and the onset of sexual maturity, were studied. For these experiments, 32 female chicks had the posterior lobe removed at six weeks of age. The same number underwent a sham operation and a similar number was retained as a group of unoperated controls. Appropriate comparisons between these groups showed no difference in rate of growth or in feed consumption during a period of 28 days following the operation.

Twenty chickens were taken at random from each of the three lots described in the previous paragraph and placed into individual laying cages shortly before the expected onset of sexual maturity. The three groups commenced to lay within a few days of each other and all three groups laid with equal intensity during the first 34 days after the first egg was laid. Statistical analysis showed no differences in either the age at the onset of sexual maturity nor in the intensity of laying between the three groups.

In further experiments the effects of posterior lobe removal on the weight and the histology of various glands and on the reproductive organs, were

studied. In eight birds the posterior lobe was removed at eight weeks of age while another eight females underwent a sham operation and eight birds served as unoperated controls. Four weeks later when the animals were 12 weeks old, they were killed. There were no significant differences in body weight, adrenal, thyroid, ovary or oviduct weights between the three groups at the time of autopsy.

In the groups described above red blood cell counts and hematocrit determination were also made. These observations were also made on one additional group of chickens which were operated at the age of 11 weeks. Because the red blood cell counts and hematocrit values were obtained at the age of 12 weeks, comparisons were available between birds which had lived without the posterior lobe of the pituitary gland for 30 days and for 7 days respectively. It is seen (Table 1) that no significant differences in

TABLE 1. EFFECT OF NEUROHYPOPHYSECTOMY ON RED BLOOD CELL COUNT AND HEMATOCRIT

Treatment	No. of birds	RBC millions/mm.^{3} Mean ±SD	Hematocrit per cent Mean ±SD
Neurohypophysectomy			
1 month	8	2.58 ±0.18 (1)	29.6 ±2.84
1 week	8	2.56 ±0.25 (2)	28.3 ±1.41
Sham operation	8	2.33 ±0.21 (3)	30.0 ±1.94
Unoperated controls	8	2.32 ±0.16 (4)	29.0 ±1.24

Significance levels:
1 vs 3—5% vs 4—2%
2 vs 3—1% vs 4—5%

hematocrit values were observed between the treatments. However, one week and four weeks after the operation the red blood cell counts were significantly elevated while in the sham operated group these values remained normal.

In subsequent experiments it was of interest to establish the effects of removing the posterior lobe of the hypophysis on the reproductive cycle of sexually mature laying hens. The hens used belonged to the White Leghorn breed which were in full and continuous production at the time of operation. Eight hens were subjected to total posterior lobe extirpation, eight were sham operated and eight served as unoperated controls. Beginning one week before operation, daily records of water consumption and egg production, and weekly records of body weight and comb size, were made and continued throughout the experimental period of 40 days following operation.

Both operated groups ceased laying immediately following the operation. The comb sizes in these groups showed a maximum reduction of 10% in the group in which the posterior lobe was removed and of about 7% in the sham operated group. The differences in comb size between the groups were not significant but it was found that a significant though temporary depression in comb size occurred in the two operated groups. Similarly,

TABLE 2. EFFECT OF NEUROHYPOPHYSECTOMY ON EGG PRODUCTION OF HENS

Treatment	No. of birds	No. of days from operation to first egg (±SD)	Mean No. of eggs laid in first 17 days after onset of laying (±SD)
Neurohypophysectomy	8	36.5±22.2*	10.4±1.6
Sham operation	8	15.4±10.3*	11.5±1.3
Unoperated controls	8	continuous laying	10.6±1.3

* Difference significant $p = 0.2$.

the groups which had been subjected to complete posterior lobe extirpation showed a maximal decrease in body weight of 12% while the birds in the sham operated group lost about 9% in body weight. However both groups recovered the lost weight and by the fourth week after the operation had exceeded their respective pre-operative body weights. As in the case of the comb size, the post-operative decrease in body weight of the operated groups was significant in comparison to the normal controls, but the difference between the two operated lots was not significant.

Eventually both operated groups resumed egg production but the interval from operation to the first egg was more than twice as long in the group which had the posterior lobe removed as it was in the sham-operated group. However, once production was resumed, all three groups laid the same number of eggs (Table 2).

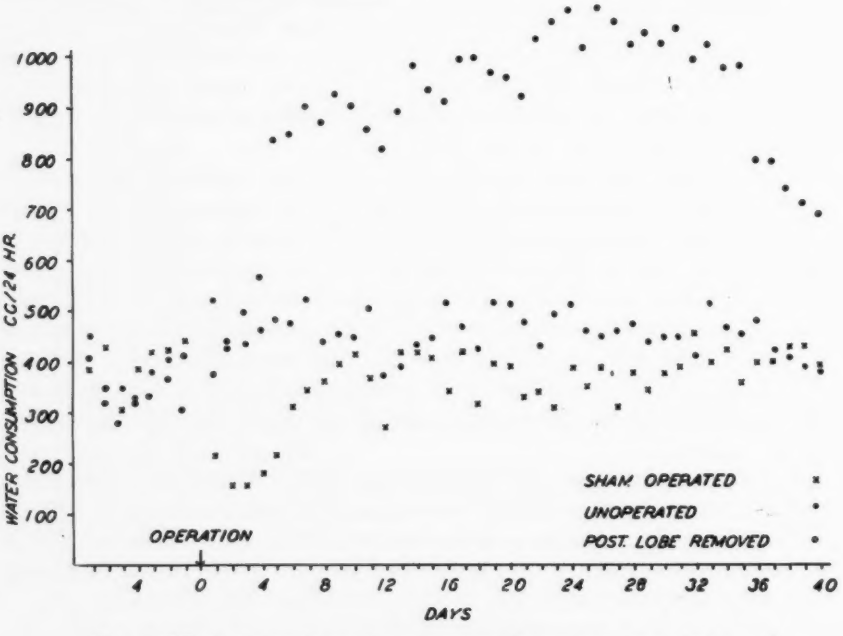

FIG. 1. Effect of neurohypophysectomy on the water intake of birds.

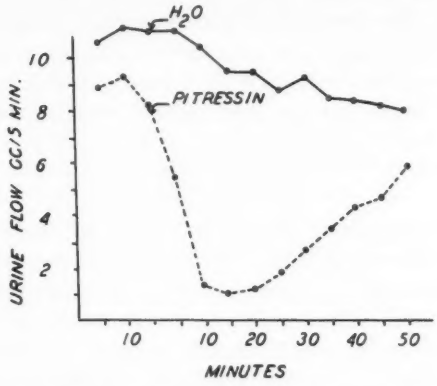

FIG. 2. Effect of pitressin on polyuria of neurohypophysectomized hens.

The water consumption showed an immediate and significant increase in the group from which the posterior lobe was removed (Fig. 1). Birds in this group drank two to three times more water during the next 34 days than did the ones in the sham operated or in the control groups. It is interesting that sham operated birds showed an immediate drastic but temporary decrease in water intake which is presumably due to surgical trauma. No such decrease was noted in neurohypophysectomized birds which apparently remained unaffected by the trauma (Fig. 1). Even though the sham operated birds consistently and throughout the experimental period drank less water than did the normal controls, this difference was not statistically significant. Polydipsia was accompanied by polyuria in neurohypophysectomized birds which could be controlled by the intramuscular injection of 0.5 units of Pitressin (Parke, Davis and Co). While the effect of

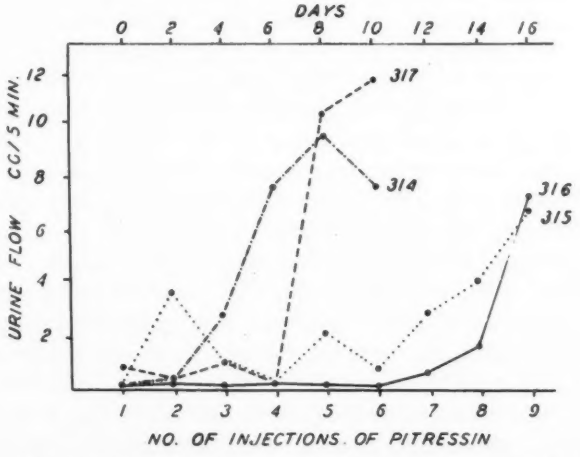

FIG. 3. Rate of development of refractoriness to pitressin.

a single injection was immediate and dramatic (Fig. 2) hens developed refractoriness to the hormone very rapidly (Fig. 3), one hen becoming refractory after the second injection (No. 314), another after four injections (No. 317), while the remaining two hens showed refractoriness after the eighth injection.

DISCUSSION

Removal of the posterior lobe of the pituitary gland does not seem to have any measurable effect on either the growth rate or the reproductive functions of chickens. It does not delay the age of sexual maturity when it is performed on immature females, neither does it diminish the rate of ovulation or oviposition after sexual maturity is reached. When the operation is performed on mature laying hens ovulations are interrupted for 30 to 40 days and comb size and body weight are reduced somewhat. Both comb size and body weight eventually return to, or exceed pre-operative levels. Similar effects but of much smaller magnitude are observed in sham-operated hens. It is thought that the interruption of ovulations is due to regression of the ovaries (hence the decrease in comb size), and the depression in body weight is due to the surgical trauma and that these effects are not due to the absence of posterior lobe secretions.

Because it is generally assumed and stated that oviposition in the hen is controlled by the hormones of the posterior pituitary, it was surprising to find that the removal of the posterior lobe had no effect on oviposition. In fact, there appeared to be no effect whatever on the motility of the oviduct and the shell gland since the total time spent by the forming egg in the various portions of the duct was not increased. The eggs laid were normal in size and shell texture. While these observations suggest that the act of oviposition does not depend on uterine contractions initiated by the oxytocic principle, the possibility remains that such an interdependence does exist but that the oxytocin comes from a source other than the neurohypophysis. In view of the work of Scharrer (1952), Scharrer and Wittenstein (1952) and others, it is possible that the neurosecretory cells of the nuclei supraoptica and paraventricularis, which have been shown to produce the hormones which are stored in the neurohypophysis, provide the oxytocin necessary to initiate uterine motility in absence of the posterior lobe. Whether the rate of oxytocin secretion by the hypothalamus is variable or constant remains unknown.

The one constant effect of neurohypophysectomy, which chickens share with mammals, is the immediate onset of sustained and prolonged polydipsia and polyuria, to neither of which an adjustment occurs within the first 30–40 days after the operation. While these conditions can be adequately and dramatically controlled with Pitressin, the rapidity with which complete refractoriness to Pitressin sets in (2–8 injections) is of interest and lacks adequate explanation. It seems extremely unlikely that the refrac-

toriness is due to the formation of antibodies even after as many as eight injections in as many days.

SUMMARY

Neurohypophysectomy in immature female chickens had no effect on growth rate, amount of feed consumed and the weights of the thyroids, adrenals, ovary or oviduct.

When this operation was performed in mature laying hens, loss in body weight and cessation of ovarian function was attributed to surgical stress. After recovery from the latter, body weights returned to normal, ovulation and oviposition occurred at the same rate as they did in unoperated controls. The possibility remains that oxytocin is essential in initiating expulsion of the egg from the shell gland but in neurohypophysectomized birds this hormone may come from the hypothalamus.

Neurohypophysectomy resulted in a significant increase in the number of red blood cells (per mm.^{3}) and led to marked polydipsia and polyuria. The latter could be controlled by Pitressin but complete refractoriness to this hormonal preparation occurred remarkably rapidly i.e., after two to eight injections.

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THE EFFECTS OF THE ADRENAL CORTEX ON THE RELEASE OF N^{15} FROM TISSUE PROTEINS OF RATS PREVIOUSLY FED N^{15}-GLYCINE

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SEVERAL recent investigations (White *et al.*, 1948; Hoberman, 1953; Clark, 1953; Parson *et al.*, 1952) have been concerned with the effects of the adrenal cortex on the incorporation of N^{15}-glycine into tissue proteins under various conditions, from which attempts have been made to assign to the C^{11}-oxysteroids the role of increasing catabolism, decreasing anabolism, or both. These experiments were all designed in such a way that the endocrine excess or deficiency existed prior to or concomitant with the administration of labeled glycine. The possibility therefore existed that the observed results were secondary to the metabolic state previously established by the endocrine disturbance, namely an increased net mobilization of endogenous foodstuff from the periphery in the case of cortisone-treated animals (Wells, 1940; Wells and Kendall, 1940; Silber and Porter, 1953); or a decreased net mobilization in the case of adrenalectomized animals (Wells and Kendall, 1940). Probably the prior existence of either general state, i.e., a positive or negative nitrogen balance, would alter the manner in which additional exogenous substrate is metabolized (Handler, 1952). It was therefore necessary to differentiate between the effects of adrenal cortical steroids upon incorporation of labeled amino acids into tissue proteins, and their possible effects on the release of label which had been incorporated into tissue proteins prior to establishing an endocrine imbalance.

An approach for observing the latter phenomena was provided by Shemin and Rittenberg (1944) who measured the rate of change of isotope concentration in various tissue proteins of animals which previously had received identical quantities of N^{15}-glycine. The present variant explored in an extension of these experiments was the establishment of an adrenal cortical excess or deficiency immediately after the last N^{15}-glycine feeding. Analaysis of the data obtained from these control, adrenalectomized and cortisone treated animals indicate that the contentions made by previous workers (Clark, 1953; Parson *et al.*, 1952) that adrenalectomy increases

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protein synthesis while cortisone decreases it, are equivocal. The increased percentage excretion of isotope found in starved rats given cortisone could be accounted for as an increased breakdown of protein, and it was not necessary to invoke the supposition that cortisone inhibited protein synthesis. In addition, the data indicate that administration of cortisone was associated with a net increase in liver proteins under the conditions of these experiments.

EXPERIMENTAL METHODS AND PROCEDURES

The general treatment of the experimental animals, the procedures for administration of the N^{15}-glycine, and the methods of analysis for protein nitrogen and N^{15} concentrations have been reported in a previous paper (Fritz, 1955). Briefly, 150 mg. of 31.9 atom per cent excess N^{15}-glycine was administered orally in six separate feedings over a period of three days to male Sprague Dawley rats weighing between 180–220 gm. During this time and for approximately two weeks previously, the animals were maintained on a 15% casein diet[2] *ad libitum*. As soon as the N^{15}-glycine feedings were begun, rats were housed in separate metabolic cages and urinary nitrogen excretion was determined for the remainder of the experimental period. On a few rats fecal nitrogen excertion was also measured. Until the last N^{15}-glycine feeding was completed, all rats were treated identically. At this time (called time 0), the animals were separated into three groups of rats which included normal control, adrenalectomized, and cortisone-treated animals. These animals were subsequently starved, and were sacrificed at varying periods of time up to 161 hours after the last N^{15}-glycine feeding.

Immediately after the last N^{15}-glycine feeding, one group of rats was anesthetized with nembutal, and each was bilaterally adrenalectomized. The adrenalectomized rats were provided with a one per cent solution of sodium chloride in the drinking water, and were given no other replacement therapy.

In a second group of rats, each animal received 5 mg. per day of cortisone acetate intramuscularly, with the initial injection being given 18 hours after the last N^{15}-glycine feeding.

The urine was collected at 24 or 48 hour intervals from the metabolic cages, and was analyzed for total nitrogen. During the interim periods, toluol was present as a preservative in the collection flasks.

RESULTS

Nitrogen balance studies

Since all animals used in this study were starved from the last N^{15}-glycine feeding until sacrifice, the urinary nitrogen excretion is equal to the negative nitrogen balance during this period. As would be expected (White, 1949), cortisone treatment greatly augmented the negative nitrogen balance, while adrenalectomy diminished nitrogen excretion during the later stages of the experimental period. The first section of Figure 1 summarizes these findings.

From a net standpoint the cortisone-treated rats excreted approximately 100 mg. nitrogen per day more than normal fasted controls. In addition

[2] Percentage constituents of this diet were casein—15%; corn starch—47%; lard—23%; Wesson oil—4%; cod liver oil—2%; Brewers yeast—5%; Osborne-Mendel Salts #1 obtained from Fisher Scientific—4%.

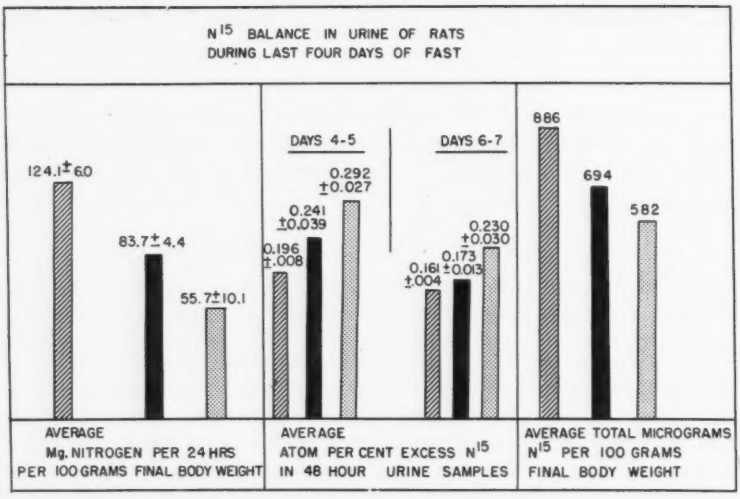

FIG. 1. Diagonal bars represent cortisone-treated rats; solid bars represent normal rats; and dotted bars represent adrenalectomized rats. Average figures plus or minus standard deviation from the mean are cited above the respective bars. The number of animals used in each group were 5 adrenalectomized; 8 cortisone-treated; and 22 normal rats. All animals were fasted for a total of 161 successive hours after the last N^{15}-glycine feeding.

there was a redistribution of body proteins, especially in the case of liver. The data in Table I clearly show that the livers from cortisone-treated animals did not lose as much protein nitrogen as did the livers from the other two groups of rats. At the end of a week's starvation the cortisone-treated rats had livers containing as much protein nitrogen as after 18 hours of starvation, while the livers of untreated and adrenalectomized starved

TABLE 1. TOTAL PROTEIN NITROGEN CONTENT OF ORGANS FROM RATS FASTED FOR VARYING PERIODS OF TIME

Fasting time in hours	18	90		161		
Condition of animals	Normal	Normal	Cortisone treated	Normal	Cortisone treated	Adrenalectomized
Liver	*106.0±10.6* (12)	*79.5±12.1* (6)	*87.3 ±2.5* (4)	*69.3 ±8.2* (16)	*102.4 ±11.7* (9)	*61.3±8.5* (5)
Spleen	*18.4± 2.5* (11)	*11.1± 5.8* (4)	*7.6 ±1.8* (4)	*8.8 ±1.3* (16)	*7.1 ± 1.9* (4)	*12.2±2.1* (5)
Thymus	*3.7± 0.97* (11)	*1.5± 0.38* (4)	*0.66±0.25* (4)	*1.21±0.33* (16)	*0.40± 0.14* (4)	*5.4±0.46* (5)
Kidney	*17.5± 2.0* (5)	*16.4± 1.2* (4)	*15.1 ±0.6* (4)	*15.6 ±1.0* (11)	*14.6 ± 2.1* (4)	—
Heart	*8.5± 1.4* (5)	*8.4± 1.7* (3)	*7.6 ±0.5* (4)	*7.5 ±0.3* (11)	*8.0 ± 0.6* (4)	—
Testis	*15.7± 2.0* (4)	*15.6± 1.9* (3)	*15.5 ±0.2* (4)	*14.5 ±2.2* (3)	*13.7 ± 0.5* (2)	—

Italicized numbers indicate average mg. protein nitrogen per 100 gms. initial body weight ± standard deviation from mean.
Numbers in parentheses indicate number of rats used in each group.

rats were reduced by over 30% of initial levels, in agreement with the findings of Addis and Poo (1936).

The net losses of lymphoid proteins, particularly from the thymus, were markedly different among the three groups of animals, with greatest losses occurring in the cortisone-treated rats and least in the adrenalectomized rats, in agreement with previous reports (White, 1949). Table I summarizes some of these findings. In contrast, the total protein nitrogen content of organs which lost little protein during starvation (heart, kidney, testis) remained essentially the same in cortisone-treated and in control rats, as shown in Table 1. The data of Figure 1 and Table 1 indicate that although the cortisone-treated rats excreted more urinary nitrogen than control or adrenalectomized fasted animals did, the individual total organ proteins of these animals changed independently in one of three ways: namely, an increased net loss of protein nitrogen in the case of thymus; no net change in the cases of heart, kidney and testis; and a decreased net loss in the case of liver. The few data available from adrenalectomized rats starved for a week indicate that while total thymus protein nitrogen was apparently increased, and spleen protein nitrogen was retained to a greater extent than in the other two groups, liver proteins were depleted as much as those of the control animals, in spite of the diminished urinary nitrogen excretion during the last four days of the experimental period.

Percentage of administered N^{15} excreted in urine

The cortisone-treated animals excreted approximately 55% of the administered isotope during the entire experimental period, while normal control animals excreted only 35% during the same period (Fig. 2). The data depicted in Figure 1 demonstrate that this difference is primarily a resultant of the total increased nitrogen output induced by cortisone, since the N^{15} concentrations of urine excreted by normal controls is actually of a higher enrichment than that of the cortisone-treated rats. Adrenalectomized rats excreted as much of the administered N^{15} as the control animals during the first three days of starvation, and slightly less than normal rats during the last four days of the experiment (Fig. 1). While the total nitrogen output of adrenalectomized rats was least, the average urinary N^{15} concentrations were highest, accounting partially for the unexpected relatively high percentage excretion of the administered N^{15}. The differences in urinary N^{15} concentrations among the three groups of animals probably reflect in some manner the mechanisms by which recently "synthesized" protein is catabolized by the organism.

Release of N^{15} from tissue proteins

Table 2 summarizes the N^{15} concentrations of various tissue proteins from normal, cortisone-treated, and adrenalectomized rats fasted for periods up to 161 hours. For a given set of proteins, the rate of decrease in

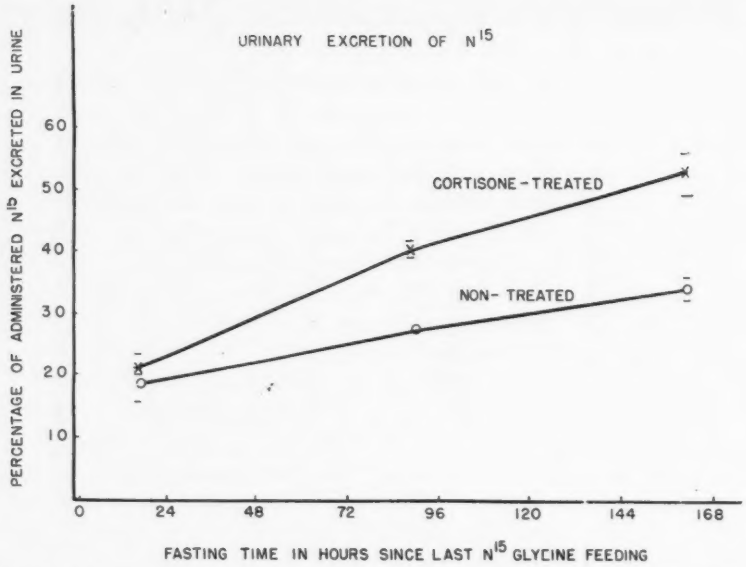

FIG. 2. The average percentage excretion of administered N^{15} is plotted for 22 normal and 8 cortisone-treated rats. The horizontal bars represent the standard deviation from the mean.

N^{15} concentrations is qualitatively similar, and there are no large quantitative differences among the three groups for any tissue proteins examined, except in the case of thymus. It should be noted that in all groups of rats, the relative distribution of a particular tissue protein N^{15} concentration

TABLE 2. N^{15} CONCENTRATIONS OF TISSUE PROTEINS OF RATS STARVED FOR VARYING PERIODS OF TIME

Fasting time in hours	18	90		161		
Condition of animals	Normal	Normal	Cortisone treated	Normal	Cortisone treated	Adrenalectomized
Plasma Proteins	*0.327±0.039* (19)	*0.231±0.014* (4)	*0.241±0.024* (3)	*0.187±0.023* (24)	*0.177±0.014* (4)	*0.196±0.019* (5)
Liver	*0.272±0.025* (19)	*0.220±0.017* (4)	*0.247±0.006* (4)	*0.188±0.014* (24)	*0.183±0.013* (4)	*0.206±0.008* (5)
Biceps Femoris	*0.065±0.007* (18)	*0.061±0.008* (3)	*0.060±0.007* (4)	*0.054±0.008* (16)	*0.056±0.011* (4)	*0.068±0.005* (5)
Kidney	*0.229±0.031* (4)	*0.192±0.011* (4)	*0.199±0.037* (4)	*0.158±0.008* (4)	*0.164±0.013* (3)	—
Spleen	*0.184±0.036* (5)	*0.138±0.012* (4)	*0.165±0.005* (4)	*0.124±0.014* (11)	*0.122±0.010* (4)	—
Thymus	*0.314±0.040* (5)	*0.217±0.027* (4)	*0.196±0.007* (4)	*0.175±0.024* (11)	*0.114±0.005* (4)	—
Heart	*0.116±0.013* (5)	*0.119±0.007* (3)	*0.131±0.010* (4)	*0.109±0.005* (3)	*0.120±0.009* (4)	—
Testis	*0.101±0.014* (4)	*0.105±0.005* (2)	*0.117±0.008* (2)	*0.101±0.015* (2)	*0.118±0.001* (2)	—

Italicised numbers indicate average N^{15} concentrations of total tissue proteins ± standard deviation from mean. Numbers in parentheses indicate number of rats used in each group.

was not appreciably altered with time, with the exception of plasma proteins, the N^{15} concentrations of which decreased more rapidly than the activity of liver proteins. The rapid loss of N^{15} from tissue proteins of high initial activity and the slow relative loss of N^{15} from tissue proteins of low initial activity occurred to comparable degrees in all three groups of animals. At the end of 161 hours of starvation, the difference in activities between the tissue proteins of highest and lowest N^{15} concentrations was smaller than the difference at 18 hours, and the extent of this difference was the same in both cortisone-treated and control rats.

Only five of twelve adrenalectomized animals used were able to survive the stress imposed by starvation for seven days. The available data, listed in Table 2, can be compared with those obtained from normal and cortisone-treated rats. It appears that there are no statistically significant differences between the N^{15} concentrations of the tissue proteins examined in this group and those of the normal and cortisone-treated rats. A preliminary report (Fritz, 1953) indicating that the N^{15} concentrations in tissue proteins of adrenalectomized animals were higher than normal was proved erroneous when more data from normal animals were collected. However, it can be seen that the *average* N^{15} concentrations of liver, plasma, and muscle protein of adrenalectomized rats are higher than those of normal rats, even though the differences are not statistically significant. One group of 5 rats was subjected to sham adrenalectomies after having received N^{15}-glycine in the same manner as the other groups of animals. The data are not included, because they were essentially the same as that found in control rats.

DISCUSSION

The results have demonstrated that among the three groups of rats examined under the conditions of these experiments, there were no significant differences in the relative rates of release of N^{15} from tissue proteins of rats previously given identical quantities of N^{15}-glycine and subsequently subjected to starvation. Of the tissue proteins examined, only thymus lost relatively more N^{15} in the cortisone-treated rats. These data are in disagreement with conclusions offered in reports by others (Hoberman, 1950; Clark, 1953) concerning the action of adrenal cortical steroids on protein metabolism. Most of the previous investigations have relied primarily on urinary nitrogen data, but two (White *et al.*, 1948; Clark, 1953) have been concerned with N^{15} concentrations in tissue proteins. In both these reports, the experimental conditions of adrenalectomy or extra steroid administration were established *prior* to feeding the N^{15}-glycine. Reconciliation of these various reports is therefore not impossible, and it appears that the existing contradictions can be resolved by reinterpretation of the available data.

The agumented percentage excretion of administered isotope by cortisone-treated animals occurred independent of whether the labeled amino

acid was administered prior to the injection of cortisone (Figs. 1 and 2), or after the animals had already received C-11 oxysteroids (Hoberman, 1950; Clark, 1953; Parson *et al.*, 1952). It therefore appears that irrespective of whether incorporation and release, or only release, of exogenous isotope by tissue proteins is being studied, the administration of cortisone increases percentage excretion of the administered isotope. It should be further noted that the N^{15} concentrations of the urinary nitrogen tended to be lowest in the cortisone-treated animals (Fig. 1), indicating that the general nitrogenous metabolic pool of these rats contained relatively more non-labeled constituents. In contrast, adrenalectomized rats, which excreted least total nitrogen, had urine with the highest N^{15} concentrations. Comparable data were also recorded in Tables 1, 2, and 13 of Hoberman's report (1950), in which the average urinary N^{15} concentrations during the first 48 hours of starvation for normal, adrenalectomized, and adrenalectomized rats given cortisone were 0.204, 0.236, and 0.187 atom per cent excess, respectively. This suggests that the more labile proteins—i.e., those which have a high rate of turnover and which were most recently synthesized—are more readily converted to urea in the absence of the adrenal gland than are the less labile proteins.

The data discussed to this point are generally agreed upon, even if interpretations of them are questionable. However, the findings that various tissue protein N^{15} concentrations were not significantly changed after adrenalectomy or cortisone administration conflict with previous reports (White, 1948; Clark, 1953). The following working hypothesis is an attempt to account for these discrepancies.

If an organism were in pronounced negative nitrogen balance for any reason, there would be a net mobilization of proteins from the periphery to the liver, where ultimate formation of urea would occur. The administration of free amino acids during this period would result in an increased probability of immediate deamination rather than incorporation into tissue proteins, provided the administered amino acid mixed freely with the non-protein nitrogen metabolic pool. The diminished incorporation of fat precursors into depot fats during starvation (Boxer and Stetten, 1944) and the impaired lipogenesis during starvation (Lyon *et al.*, 1952) are examples of comparable phenomena. It has also been shown that the negative nitrogen balance following irradiation is accompanied by a diminished uptake of labeled amino acids (White), independent of the adrenal cortex.

In contrast, if an organism were in marked positive nitrogen balance, as during growth, the administration of free amino acids during this period would result in an increased probability of incorporation into tissue proteins rather than deamination. The higher uptake of tagged glycine and alanine by foetal liver homogenates (Winnick, 1950) may be considered as direct substantiation for this assertion.

It is well known that cortisone administration is associated in some manner with an increase in net mobilization and catabolism of carcass pro-

teins (Silber and Porter, 1953); and that adrenalectomy is associated with a decrease in net endogenous mobilization and catabolism of carcass proteins (Wells, 1940; Wells and Kendall, 1940). It is therefore tempting to hypothesize that the low N^{15} concentrations of peripheral tissue proteins in cortisone-treated rats were observed previously (Clark, 1953) because a hyperadrenal cortical state was established in animals *prior* to the administration of N^{15}-glycine. Conversely, a high uptake of N^{15}-glycine by tissue proteins of adrenalectomized animals (White *et al.*, 1948) could result indirectly from the decreased endogenous mobilization of proteins. The generalized shifts in net nitrogen balance induced by the endocrine disturbance would thus result in non-specific changes in rates of isotopec incorporation into tissue proteins, as outlined above.

Results obtained in this study show that the primary differences in protein metabolism among normal, adrenalectomized, and cortisone-treated rats were related to changes in net organ protein nitrogen content. The shifts in N^{15} concentrations of various tissue proteins seemed to be surprisingly independent of shifts in the net nitrogen content. It is therefore unlikely that much insight will be gained into the mechanism of action of adrenal cortical steroids on protein metabolism by analyzing only the urinary N^{15} excretion after the administration of N^{15} labeled amino acids. The "metabolic pools" are too multiple and complex to permit adequate simplifications, such as those advanced by Hoberman (1950) and used by other investigators (Clark, 1953; Bartlett and Gaebler, 1952) in attempts to delineate the mechanism of action of hormones on metabolic processes. These approaches have been criticized elsewhere (Tarver, 1952; Reiner, 1953).

A most striking finding in net organ protein nitrogen changes with starvation was the lack of decrease in liver proteins of cortisone-treated rats after a seven day fast. Silber and Porter (1953) recently reported a similar phenomenon in cortisone-treated animals maintained on a low protein diet. It appears likely that the administration of adrenal cortical steroids to fasting animals increased the mobilization of protein from the periphery to the liver at a rate faster than the liver could adequately catabolize, leading to a net increase in the total proteins in the liver. In spite of this, the N^{15} concentrations of all cytological components of liver proteins tested (Fritz, 1955) were in the same range as that of liver proteins from normal starved animals, even though the total liver nitrogen was considerably lower in normal fasted rats. This suggests that the peripheral proteins mobilized in cortisone-treated rats had a higher N^{15} concentration than that of average carcass proteins. This possibility is examined in an accompanying paper (Fritz, 1955).

SUMMARY

In normal animals previously fed N^{15}-glycine under standardized conditions, the subsequent administration of cortisone increased the percentage

excretion of isotope, thereby indicating that cortisone enhanced protein catabolism during starvation. Neither adrenalectomy nor cortisone treatment markedly influenced the rate of change of N^{15} concentrations of various tissue proteins from rats fasted for varying periods of time. In contrast, net shifts in total protein nitrogen among selected tissues and organs were markedly influenced by adrenalectomy and cortisone treatment. The findings were discussed, and an attempt was made to reconcile discrepancies between the present data and previous reports. It was suggested that administration of cortisone to starving animals resulted in a protein mobilization from the periphery to the liver at a rate faster than the liver could catabolize it.

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THE PREFERENTIAL CATABOLISM OF RECENTLY SYNTHESIZED PROTEIN DURING STARVATION

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BY FOLLOWING the heavy nitrogen concentrations in tissue proteins from rats which had previously been fed N^{15}-glycine and maintained on a low protein diet, Shemin and Rittenberg (1944) demonstrated that the N^{15} concentration of liver proteins decreased rapidly with time, while that of carcass proteins increased. These authors subsequently postulated that the N^{15} concentration of any tissue protein would approach an "average isotope concentration," which was the calculated specific activity if the incorporated N^{15} were homogeneously distributed through all tissue proteins. This concept inferred that a decrease in N^{15} concentration of relatively highly labeled tissue proteins resulted primarily from a dilution by nitrogen transferred from other, less enriched tissue proteins, all of which interacted with constituents of a general protein metabolic pool.

The present study, undertaken initially to examine the effects of the adrenal cortex on protein metabolism, has demonstrated that under conditions which produce a marked negative nitrogen balance, the N^{15} concentrations of various tissue fractions having an activity lower that the "average isotope concentration" do not rise, but may remain constant or fall with time. These facts, in conjunction with deductions from data obtained on mobilization of proteins from the periphery to the liver under these conditions, have led to the concept that preferential catabolism of recently "synthesized" protein probably occurs during conditions of a negative nitrogen balance.

EXPERIMENTAL METHODS

Identical procedures to these, reported previously (Fritz, 1956), were used in the present study. In addition livers were homogenized in isotonic sucrose and fractionated into various cytological components by the procedure of Hogeboom *et al.* (1948). Aliquots of each component were analyzed for percentage nitrogen content and N^{15} concentrations.

Non-protein nitrogen, as used here, indicates all nitrogenous material extractable with cold 5% trichloroacetic acid.

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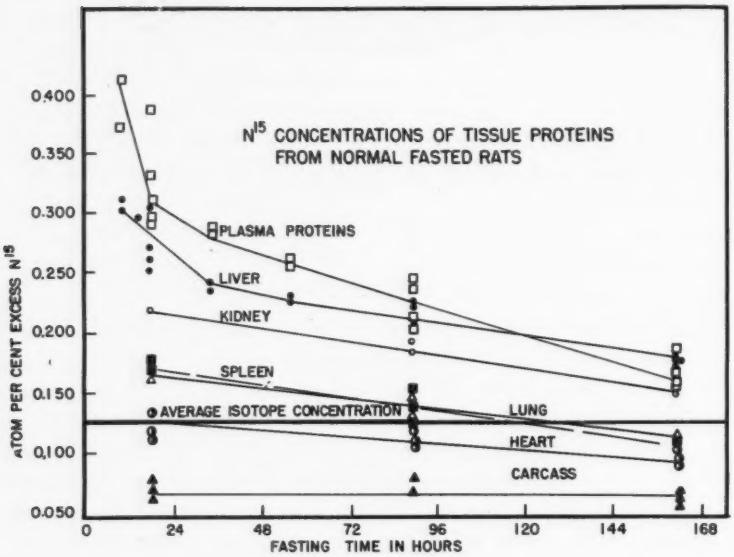

FIG. 1

RESULTS

Figure 1 indicates the general distribution of N^{15} in tissue proteins, and the changes in N^{15} concentrations with fasting time. The data are the same as those reported in Table 2 of the preceding paper (Fritz, 1956). In addition, the "average isotope concentration" (AIC) was calculated for the point at 18 hours, assuming a uniform distribution of the retained N^{15} among all nitrogenous compounds of the body. The straight line parallel to the abscissa drawn through this point is not correct, since the AIC would constantly decrease as urinary nitrogen, having a higher N^{15} concentration than the AIC, was excreted. However, the straight line for the AIC serves the purpose of facilitating a comparison of changes of tissue protein and non-protein N^{15} concentrations.

It should be noted that no tissue protein fractions listed had an increased N^{15} concentration after fasting. Exceptions to these proteins include those of hair and teeth. The rise in N^{15} concentration of dentinal proteins of

TABLE 1. N^{15} CONCENTRATIONS OF HAIR PROTEINS FROM RATS STARVED FOR VARYING TIME INTERVALS

Length of fast in hours	18	90	161
Atom Per Cent	0.013 (2)*	0.021 (2)	0.042 (2) 0.036 (1)
Excess N^{15}	0.012 (2)		0.051 (1)

* Numbers in parentheses indicate number of rats pooled for each analysis.

continually erupting rat incisors has been reported elsewhere (Fritz and Burnett, 1953). Table 1 has data on the N^{15} concentration changes of proteins obtained from hair shaved from the scapular regions of rats. However, no other carcass proteins examined showed elevated N^{15} concentrations.

Figure 2 summarizes data on the changes in N^{15} concentrations of non-protein nitrogen fractions from carcass, which consisted of all residual muscle and bone, and various muscles. Comparison with Figure 1 indi-

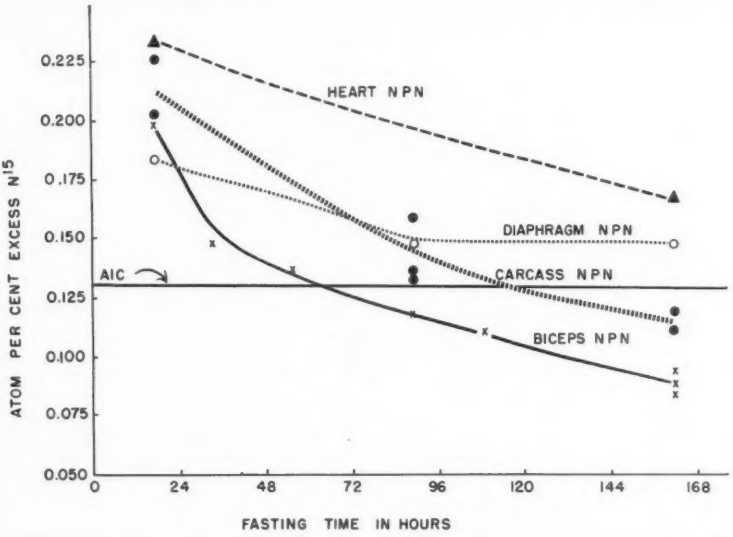

FIG. 2. Non-protein nitrogen N^{15} concentrations of muscles from rats fasted for varying periods of time. Each point represents the average determination for at least two separate animals.

cates that the NPN N^{15} concentration is always greater than the corresponding tissue protein N^{15} concentration, in agreement with the original observations by Shemin and Rittenberg (1944). It should be noted that carcass and biceps femoris NPN isotope activities decreased rapidly throughout the experimental period, eventually becoming lower than the original average isotope concentration. It is of interest that the N^{15} concentrations of NPN fractions decreased more rapidly than the N^{15} concentrations of corresponding tissue proteins, even though the absolute values were always higher for the former.

Since it was known from earlier work (Fritz, 1956; Silber and Porter, 1953) that the livers of cortisone-treated animals in negative nitrogen balance did not lose as much protein as did normal starved animals, it was decided to analyze the liver proteins of a few rats from these two groups for possible differences in N^{15} concentrations of various cytological com-

ponents obtained by differential centrifugation (Hogeboom *et al.*, 1948). Figure 3 demonstrates that although the N^{15} concentrations of total liver proteins of cortisone-treated rats were not less than those of normal rat livers, the average total quantity of N^{15} present in livers from the former animals was greater than that in livers from normal rats. This difference is primarily a reflection of the larger net nitrogen content of livers of cortisone-treated, starved rats (Fritz, 1955). Table 2 summarizes the N^{15} concentrations of various cytological components of livers from these two groups of animals. Microsomal, soluble fractions, and the mitochondrial

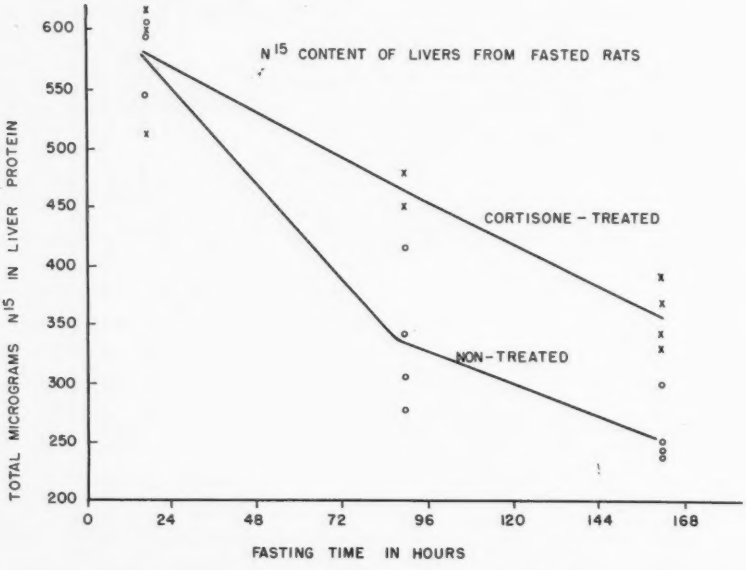

FIG. 3. Each point represents an individual rat liver. The numbers were calculated by multiplying the N^{15} atom per cent excess by the micrograms of nitrogen present in total liver proteins. Lines are drawn through average values.

fractions had highest activities, while that portion of the liver homogenates which were spun down at $700 \times g$ for five minutes (cell debris, nuclei, and red blood cells) had the smallest N^{15} concentrations. The slight shifts in relative activities of various fractions with time were parallel in both groups. The absolute N^{15} concentrations of all components in livers from two cortisone-treated rats starved for 90 hours were greater than those of untreated rats, while no absolute differences were evident between the two groups of rats fasted for 161 hours.

A comparison between the percentage loss of total tissue protein nitrogen during the seven day fast and the percentage decrease in initial N^{15} concentrations of the same tissue protein is presented in Table 3, calculated from data presented in the previous paper (Fritz, 1956). No con-

TABLE 2. N^{15} CONCENTRATIONS OF CYTOLOGICAL COMPONENTS OF LIVERS FROM RATS STARVED FOR VARYING PERIODS OF TIME

Fasting time in hours		Normal			Cortisone-treated	
		18	90	161	90	161
Total number of animals analyzed		3	2	6	2	2
Fraction	Average percentage nitrogen	Atom per cent excess N^{15}*				
Nuclear Sediment	26.42 ± 2.72†	0.263 0.255 0.188	0.183 0.192	0.139 0.151 0.181 0.175 — —	0.207 0.203	0.178 0.161
Mitochondria	18.44 ± 2.44†	0.311 0.294 0.231	0.214 0.217	0.191 0.190 0.224 0.236 0.234 0.234	0.253 0.250	0.205 0.185
Microsomes	19.72 ± 2.40†	0.337 0.322 0.268	0.224 0.229	0.192 0.188 0.229 0.226 0.230‡	0.264 0.260	0.207 0.181
Soluble Supernatant	36.80 ± 1.99†	0.359 0.336 0.265	0.219 0.221	0.186 0.181 0.218§	0.255 0.248	0.202 0.179
Total Liver	—	0.329 0.310 0.243	0.203 0.228	0.183 0.181 0.219 0.216 0.210 0.220	0.245 0.246	0.199 0.178

* The atom per cent excess N^{15} figures are cited for each rat liver analyzed. Data for a single liver are listed in the same position in each vertical column, and each set of data represents a different animal.

† Standard deviation from mean. The average percentage nitrogen figures were obtained from all 15 animals used, since the distribution was not apparently altered during starvation

‡ Livers from two rats pooled for analysis.

§ Livers from four rats pooled for analysis.

TABLE 3. THE LIABILITY OF TISSUE PROTEIN NITROGEN DURING STARVATION*

Tissue	Average percentage loss of total protein nitrogen after starvation for 161 hours		Average percentage decrease in N^{15} concentrations after starvation for 161 hours	
	Normal	Cortisone-treated	Normal	Cortisone-treated
Liver	−34.6	− 3.40	−30.9	−32.7
Spleen	−52.0	−61.5	−32.6	−33.6
Thymus	−67.5	−89.0	−44.5	−67.0
Kidney	−10.9	−16.8	−30.9	−28.4
Heart	−11.7	− 5.9	−13.8	+ 3.4
Testis	− 7.6	−12.7	0	− 1.6
Plasma Proteins	−19.5†(?)	(?)	−42.7	−45.8

* The data for this table were calculated from the data of Tables 1 and 2 of the preceding paper (Fritz, 1956).

† This figure was calculated from the data of Addis, *et al.* (1936).

sistent correlation among various tissue proteins exists between the losses in total nitrogen and total N^{15}. The net protein nitrogen loss from some tissues (kidney, heart, testis, and plasma proteins) was inappreciable, while the N^{15} concentrations of kidney and plasma proteins diminished markedly. In contrast, the net nitrogen content from the thymus and spleen decreased relatively more than did the N^{15} concentrations. In the livers of normal animals, the percentage loss of total nitrogen and of N^{15} were approximately the same. On the other hand, liver proteins of cortisone-treated rats were not appreciably diminished during starvation, while the N^{15} concentrations fell as much as in normal animals (see Table 3).

DISCUSSION

N^{15} concentrations of carcass protein and non-protein nitrogen fractions failed to rise with fasting time even though some of these concentrations were below the original average isotope concentration. This suggests that mobilization of nitrogenous components recently incorporated into metabolically active tissue proteins resulted in preferential conversion to urea rather than transfer to other tissue protein components.

Shemin and Rittenberg (1944) reported that carcass N^{15} concentrations of young, tumor-bearing animals increased while the rats were maintained on a low protein diet. In the present experiments, the absolute differences in N^{15} concentrations among various tissue proteins was less at the end of the experimental period than at the beginning, indicating an approach of activities of both highly labile tissue proteins and relatively inactive tissue proteins towards each other. Therefore, the present results differ only in a quantitative sense from those of Shemin and Rittenberg (1944) who observed the same phenomenon. Further, it should be pointed out that these authors observed no absolute rise in carcass protein N^{15} concentrations in normal, non-tumor bearing rats kept on a low protein diet for a week (Shemin and Rittenberg, 1944). In addition, "carcass proteins" included skin and hair in their measurements, and the known rise in N^{15} concentrations of hair with time (see Table 1) may have been partially responsible for the increased carcass protein N^{15} concentrations in young rats.

From the results on shifts in liver proteins of cortisone-treated animals, it appears that preferential catabolism of recently synthesized protein occurred. Since total liver protein nitrogen was greater in rats given cortisone than in starved control animals, resulting most probably from a rate of mobilization to the liver more rapid than the limiting catabolic step, the N^{15} concentrations of these proteins should be lower, since the source of mobilized proteins (carcass) had a relatively low N^{15} concentration. No cytological compartments of liver proteins tested from the cortisone treated rats had lower N^{15} concentrations than corresponding components from normal rats. One possible interpretation is that the nitrogenous substances

mobilized from the periphery were those having higher N^{15} concentrations than the average carcass N^{15} concentration. One other possible interpretation is that cortisone in some manner inhibited the catabolism of proteins initially present in the liver, thereby maintaining a high N^{15} concentration. However, this is probably not the case, since urinary excretion of nitrogen increased after cortisone treatment (Frtiz, 1956), and present knowledge indicates that urea formation occurs primarily in the liver (Baldwin, 1952).

There are other facts which suggest that amino acids recently deposited into tissue proteins are metabolically distinguishable from amino acids previously incorporated. It is well known that tissue proteins which have the highest uptake of tagged amino acids also have the most rapid rates of release of isotope (Shemin and Rittenberg, 1944). Previously, this has been interpreted as an indication of a high "protein metabolic rate" (Shemin and Rittenberg, 1944) of the tissues concerned, or a high "lability" of tissue proteins (Tarver, 1949) where "lability" was defined as the percentage loss of tissue proteins during starvation, and "protein metabolic rate" was equated with the turnover rate of tissue proteins. The data in Table 3 indicate the absence of any consistent correlation between percentage loss of total tissue proteins and rate of release of isotope from tissue proteins. This interpretation can be questioned, since pure proteins were not isolated. Both the percentage decrease in total protein nitrogen and the percentage decrease in relative isotope concentration were determined on trichloroacetic acid residues, which undoubtedly contain many different species of proteins in any single tissue or organ. However, the sustained excretion of urinary nitrogen having an isotope concentration higher than the average isotope concentration is incontestable. The only possible description from a net standpoint is that N^{15} recently incorporated into tissue proteins was preferentially mobilized and excreted. Further, the percentage excretion of recently incorporated isotope was increased as the negative nitrogen balance became more marked, as with cortisone (Fritz, 1956). This strongly suggests that preferential catabolism of recently synthesized protein does take place under conditions of marked negative nitrogen balance, but does not indicate whether whole protein species are preferentially degraded, or whether fragments of protein molecules are split off.

SUMMARY

During conditions of negative nitrogen balance, the N^{15} concentrations of all tissue proteins examined, except those of incisor dentin and hair, failed to increase with fasting time, regardless of whether the initial activity was above or below the "average isotope concentration." Differential centrifugation of livers from starved, cortisone-treated rats revealed that all fractions retained more protein nitrogen than did corresponding fractions from starved normal controls. Concomitantly, it was shown that

the N^{15} concentrations of various corresponding fractions were essentially the same in both groups of animals. It was therefore concluded that recently deposited liver protein in the cortisone-treated, starved rat was derived from nitrogenous compounds of higher N^{15} concentrations than that of average total carcass proteins. This and other data were interpreted to signify that recently "synthesized" protein was preferentially catabolized under the conditions of these experiments.

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IODINE METABOLISM IN SURVIVING HUMAN THYROID SLICES MAINTAINED IN A MEDIUM OF CONSTANT COMPOSITION[1]

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IN A previous paper (Molnar *et al.*, 1956) it has been shown that rates of I^{131} uptake by surviving human and canine thyroid slices could not be determined by the method of incubation of slices in a fixed volume of medium containing I^{131}. A new method was therefore devised in which a medium of constant composition flowed by gravity at a constant rate over thyroid slices. This method, termed "gravity-flow incubation," and its potentialities and limitations form the subject of this paper.

MATERIALS AND METHODS

Histologically normal thyroid tissue was removed by biopsy from 31 clinically euthyroid patients undergoing operations on the neck. Tissue was also obtained at thyroidectomy from four patients with exophthalmic goiter after preparation in the customary fashion with strong iodine solution (Lugol's solution). The preparation of slices and the chemical methods used in this study have already been described (Molnar *et al.*, 1956). The composition of the incubating medium differed from that used previously only in that the concentration of I^{131} was 4 μc. per 100 ml.

The tissue chamber was constructed of siliconized lucite as shown in Figure 1. Inlet and outlet tubes were identical in internal diameter with the height of the tissue chamber when a specially fitted rubber stopper was fully in place. This height was a little less than 2 mm., which did not permit movement of three tissue slices (each about 0.5 mm. thick) placed one on top of the other. The combs shown in Figure 1 also served to prevent the slices from moving or entering the tubing.

The reservoirs for the incubating fluid were round-bottomed pyrex flasks of 2 liters, capacity. Glass tubing on the bottom served as an outlet which passed through a large rubber stopper in the floor of a water bath. The glass tubing ended in a simple manifold, which consisted of a small rubber stopper pierced by two or four pieces of stainless-steel stock of 2 mm. internal diameter.

Plastic tubing connected each steel stock of the manifold with the inlet tube of one of the tissue chambers. From the outlet tube similar tubing led to a glass stopcock fitted with a standard needle adapter. The length of each tubing to and from the tissue chamber was 1 meter, allowing the vessel to be transferred from water bath to scintillation counter.

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[1] Abridgment of portion of thesis submitted by Dr. Molnar to the Faculty of the Graduate School of the University of Minnesota in partial fulfillment of the requirements for the degree of Doctor of Philosophy in Medicine. (The Mayo Foundation, Rochester, Minnesota, is a part of the Graduate School of the University of Minnesota.)

Stainless-steel three-way stopcocks interposed at the inlet of each tissue-chamber vessel enabled the vessel to be simultaneously connected to two reservoirs containing different media. The flow could be switched with ease to come from either reservoir. In each experiment four tissue-chamber vessels, leading to four outlet stopcocks, were used simultaneously.

The reservoirs were placed higher than the glass stopcocks to permit the medium to flow by gravity. The flow was regulated in part by a constant pressure of gas maintained over the medium in the reservoir. The rubber stopper of the bottle was pierced by a long piece of glass tubing leading to the bottom of the reservoir and a short piece of glass tubing leading to the outside with a rubber tube over it, so that it could be clamped. Inside the long glass tube and fitting loosely was the plastic tubing from the oxygen-

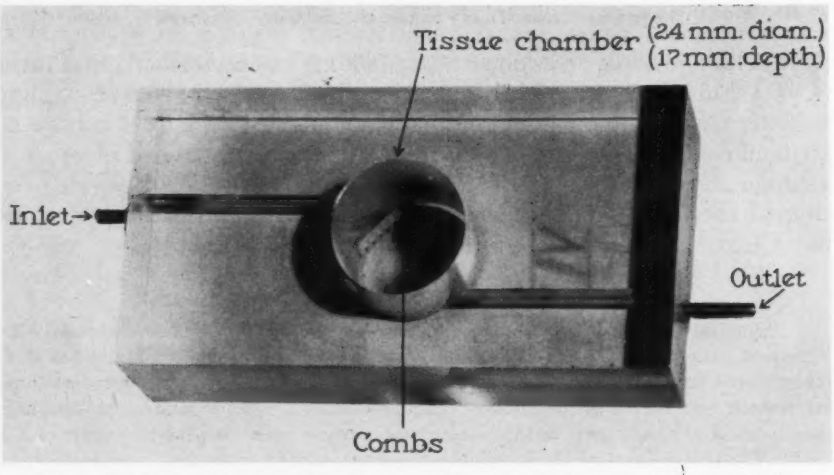

FIG. 1. Plastic vessel containing the tissue-slice chamber for gravity-flow incubation. Rubber stopper, not shown here, sits immediately on top of the combs, giving the stoppered tissue chamber a height of about 2 mm.

carbon dioxide gas tank. With the medium flowing and the outlet tube clamped, the effective pressure was that at the lower end of the long glass tube. Since this level was not reached until the end of the experiment, the effective pressure on the medium was constant. In addition, the glass stopcocks at the outlet could be adjusted to a desired rate of flow measured in drops per minute. Drop size was controlled by attaching standard-sized hypodermic needles to the adapters on the glass stopcocks. The relationship of drop size to volume was determined for each needle used.

Temperature was kept uniform with two thermostatically controlled water baths, one containing reservoirs and the other containing incubation chambers and about 90% of the plastic tubing. Radioactivity was measured with a sodium iodide (thallium-activated) scintillation counter connected to a model 1070-A Multiscaler (Atomic Instrument Co.). One tissue chamber at a time could be fitted exactly over the counter crystal. Chamber and crystal were shielded by a lead brick hollowed out for accurate positioning of the vessel and for the passage of the inlet and outlet tubings.

Preceding each experiment the medium was gassed with 95% oxygen and 5% carbon dioxide for an hour.[2] Three thyroid slices were then placed in each tissue chamber and the flow of medium was adjusted. Measurements of radioactivity were made serially for

periods of 100 seconds per tissue chamber during each experiment. Any increase in radioactivity in the chamber represented increased concentration of I^{131} in tissue. In blank runs no rise in radioactivity occurred over the chamber. Background, activity of an I^{131} standard, and rate of flow were re-checked periodically. At the end of each experiment slices were rinsed, blotted and weighed, and the I^{131} was determined in the scintillation well counter in comparable geometric position with aliquots of the original and of the effluent medium.

The serially recorded radioactivity over the tissue chamber was a simple linear function of time. The slope of this line, representing the rate of I^{131} uptake, was calculated from the relation

$$r = \frac{\bar{y} - y^0}{\bar{x}} \times \frac{1{,}000}{w}$$

where r = uptake of I^{131} in counts per second (c.p.s.).gm./minute, $\bar{y}$ = mean uptake in gross c.p.s., estimated by visual fit with y^0 = c.p.s. at x (time) zero, $\bar{x}$ = mean time and w = weight of tissue in mg.

To calculate the slope of the line resulting from changes brought about in the course of experiments—for instance, when, after a control period, either rate of flow or composition of the medium was changed—the arithmetic mean ($\bar{y}'$ and $\bar{x}'$) of the observed points on the new line was lined up by eye with the first or second actually observed point (y', x') on the line to obtain the best fitting straight line. By using the best fitting initial points, the slope was determined using the relation:

$$r' = \frac{\bar{y}' - y'}{\bar{x}' - x'} \times \frac{1{,}000}{w}.$$

RESULTS

1. *Preliminary Experiments.* The rate of uptake of I^{131} was determined in 14 experiments on slices from 12 normal and two hyperplastic glands. The flow of the incubating fluid was 0.5 ml./minute. The rate of I^{131} uptake was 2.6 ± 2.4 (S.D.) c.p.s./gm./minute. In absolute terms, the rate of uptake was 9.5 ± 8.8 (S.D.) $\times 10^{-2}$ μc./gm./hour, which represents a "clearance" of 2.32 ± 2.22 ml. of medium/hour. Per gram tissue, the slices removed 7.9% of the total I^{131} to which they were exposed. The T/M ratio (tissue-to-medium ratio) at the end of each experiment, determined by comparing slices with an aliquot of medium, correlated well with the rate of I^{131} uptake ($k = +0.93$).

2. *Effect of Flow Rate on I^{131} Accumulation.* In four experiments the rate of flow was altered after 90 minutes of observation at a flow of 0.5 ml./minute. The resulting alteration in rate of I^{131} accumulation is shown in Table 1. In general, the higher the flow rate, the more rapid was the I^{131} uptake. The coefficient of correlation between change in rate of I^{131} uptake and change in rate of flow was +0.95.

3. *Removal of Radioiodine from the Tissue by the Flowing Medium.* Slices

[2] It was estimated that adequate oxygen was available for the tissue slices. Assuming the oxygen requirement of the tissue to be about 0.60 mm.^{3}/mg. wet wt./hour (Freinkel and Ingbar, 1955), and an oxygen content of 20 mm.^{3}/ml. of medium (Umbreit *et al.*, 1951), a minimal rate of flow of 0.05 ml./min./100 mg. slice would be necessary. The slowest flow rate employed in this study represents at least 2.5 times this value.

of thyroids previously labeled with I^{131} *in vivo* were incubated with non-radioactive medium in three experiments. I^{131} was lost from the slices. When the data were plotted on semilogarithmic paper, the decrease of I^{131} was a linear function of time. The removal of tissue I^{131} was calculated using the formula

$$\lambda = \frac{\log C_{t_1} - \log C_{t_2}}{(t_1 - t_2) \log_e 2} \times 100$$

where C_{t_1} was the counts/second at time t_1 and C_{t_2} was the counts/second at time t_2. The proportional rates of loss of iodine were 0.17, 0.09 and 0.12% per minute in three experiments with normal tissue, and 0.23 and 0.28% per minute in two experiments with hyperplastic tissue. Incubation for 3 hours of slices with 10-mg. per liter thyrotropin (Armour thytropar) did not alter the proportional rate of loss. Increasing the rate of flow eightfold to tenfold after a control period at 0.5 ml./min. increased the rate of loss by about 25%.

TABLE 1. EFFECT OF RATE OF FLOW OF MEDIUM ON RATE OF I^{131} UPTAKE*

Change in rate of flow	Change in rate of uptake
8.0	2.1
8.0	2.0
5.4	1.5
5.4	2.0
12.6	2.7
14.3	2.6
8.6	1.9
8.6	1.5
28.8	4.5
28.8	3.6
0.2	0.3
0.2	0.6

* The initial flow rate and rate of I^{131} accumulation at this flow were taken as unity. The entries in Table 1 are therefore multiples or fractions of the initial rates.

4. *Effect of Iodide on the Rate of I^{131} and I^{127} Uptake.* After a control period with the standard incubating fluid (containing 0.2 μg. inorganic iodide per 100 ml.), media enriched with iodide in concentrations of 2, 10, 20, 40, 70 and 100 μg. per 100 ml. were introduced in the course of three experiments with normal tissue. Although total I^{127} uptake by the slice was increased with increasing concentration of iodide, successive increments were progressively smaller. The I^{131} uptake was diminished (Fig. 2).

5. *Effect of Iodinated Organic Compounds on the Uptake of I^{131}.* I^{131} uptake was decreased following the introduction of L-triiodothyronine, L-thyroxine and thyroglobulin (Table 2). Triiodothyronine appeared to exert the greatest suppressive effect.

6. *Effect of Antithyroid Agents on the Uptake of I^{131}.* Eighteen experiments with suitable controls were performed to determine the effect of blocking agents on I^{131} uptake of slices. Media containing 10^{-3} *M* thiouracil

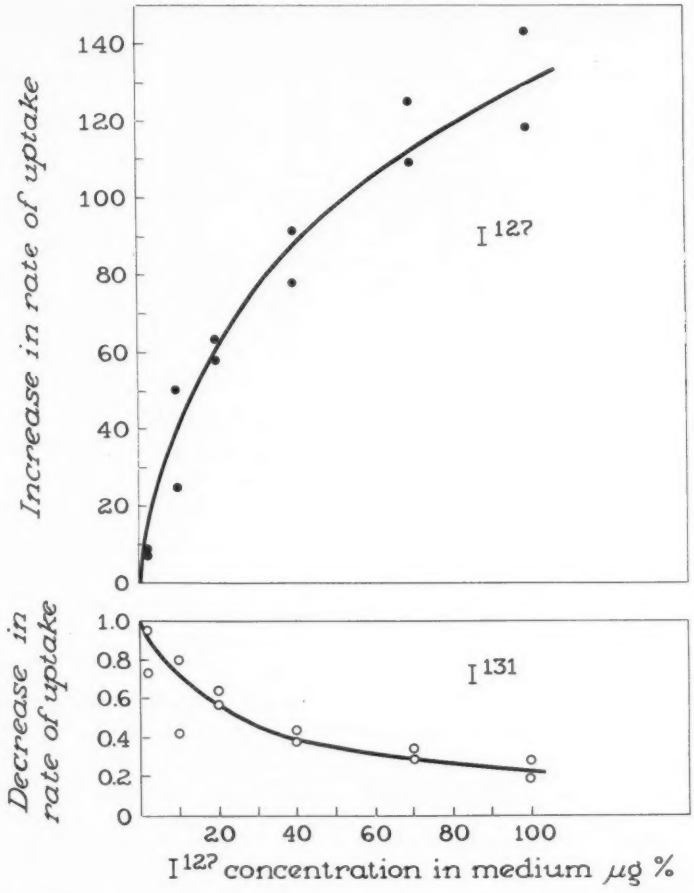

FIG. 2. Changes in I^{131} and I^{127} uptake at varying concentrations of stable iodide in the medium. Each point represents the ratio of I^{131} uptake to control I^{131} uptake (circles) or the ratio of I^{127} uptake to control I^{127} uptake.

or 10^{-3} *M* methimazole (tapazole) were employed at flow rates of 0.5 ml./min. and 4 ml./min. respectively. That these concentrations of antithyroid agents were effective is indicated by the finding that the protein-bound I^{131} of the slice was not more than 2%, whereas the protein-bound I^{131} of control slices was 75% after 180 minutes of incubation. Figure 3 shows that the pattern of I^{131} uptake in control slices is rectilinear, whereas that of the blocked slices is exponential. By means of graphic differentiation (Lipka, 1918; Nitowsky and Puck, 1952) estimates were obtained for rate of uptake and maximal (asymptotic) uptake of I^{131} for each of the blocked curves. The former may be compared directly with uptake rates calculated for control slices; the latter, divided by concentration of radio-

TABLE 2. EFFECT OF IODINATED ORGANIC COMPOUNDS ON I^{131} UPTAKE

Compound	Concentration, μg. organic iodine per 100 ml.	Decrease in rate of I^{131} uptake, %
L-triiodothyronine	2.93	32
	2.93	37
	14.65	41
	14.65	57
L-thyroxine	2.93	31
	2.93	7
	14.65	33
	14.65	24
Thyroglobulin	2.93	19
	2.93	32
	14.65	51
	14.65	34

activity in the medium, provides an estimate for T/M ratio as a measure of the ultimate magnitude of the osmotic gradient produced by the thyroid cells under equilibrium conditions. The results of such calculations are compared in Table 3. The mean rate of uptake so calculated was 3.38 ± 0.58

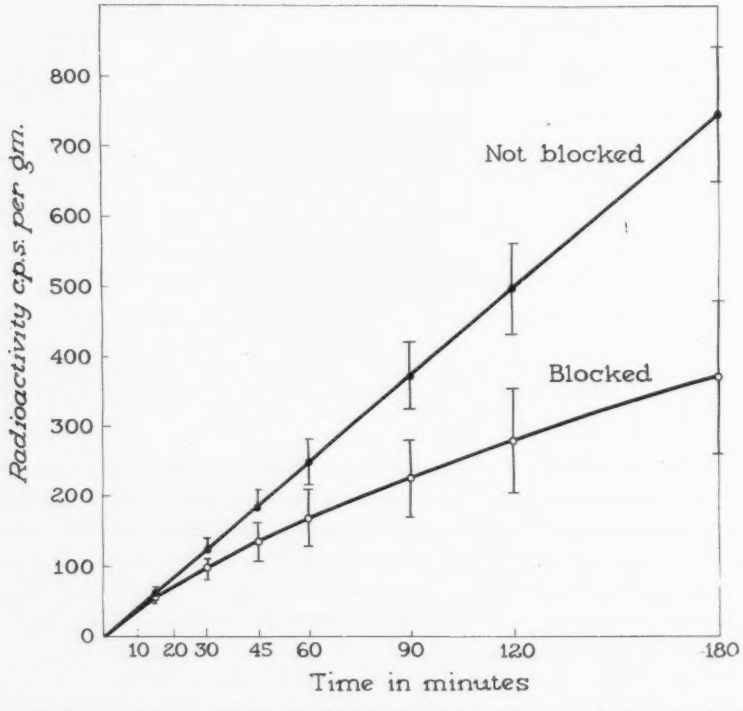

FIG. 3. Accumulation of I^{131} in blocked (4 glands) and in control slices (6 glands) of normal thyroid tissue. Each point is the mean of 10 observations. Vertical bars are the standard errors of the means.

TABLE 3. I^{131} UPTAKE IN BLOCKED AND CONTROL THYROID SLICES

Control		Blocked		
Flow rate, ml./min.	Uptake, c.p.s./gm./min.	Flow rate, ml./min.	Uptake, c.p.s./gm./min.	T/M ratio
0.5	2.43	0.5	2.18	8.7
	3.21		1.07	4.2
	3.03		1.77	6.15
	1.94		2.96	18.9
	3.29			
Mean	2.78 ± 0.26*		2.00 ± 0.39	9.5 ± 3.3
4.0	3.04	4.0	2.92	11.1
	3.45		1.95	11.2
	3.61		4.20	21.9
	4.28		4.45	26.5
	6.65			
	3.83			
Mean	4.14 ± 0.53		3.38 ± 0.58	17.7 ± 3.9

* Standard error of mean.

(S.E.) c.p.s./gm./minute at 4 ml./minute rate of flow of the medium and 2.00 ± 0.39 (S.E.) c.p.s./gm./minute at 0.5 ml./minute rate of flow. The T/M ratios at these two rates of flow of the medium were 17.7 ± 3.9 (S.E.) and 9.5 ± 3.3 (S.E.) respectively.

7. *Effect of Iodide on I^{131} and I^{127} Uptake in Blocked Slices.* The effect of media containing methimazole (10^{-3} *M*) and NaI in concentrations varying from 0.2 to 100 μg. per 100 ml. on I^{131} uptake was studied. At 20 μg. iodide per 100 ml., total I^{127} uptake was increased from a control value (standard medium) of 0.006 μg. I/gm. tissue to 0.65 μg. I/gm. tissue after 3 hours.

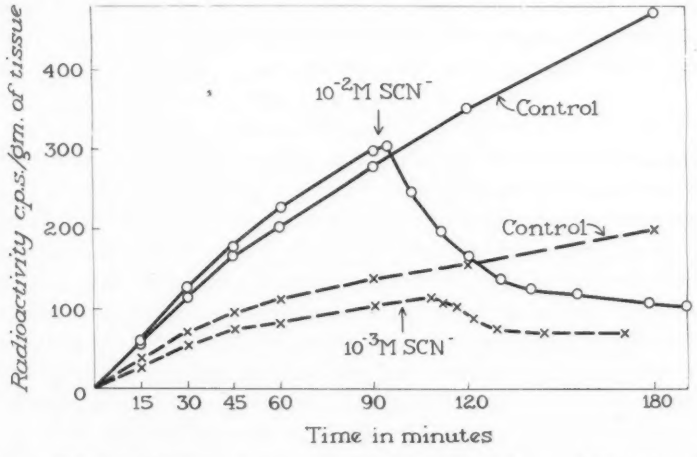

FIG. 4. Effect of thiocyanate on accumulation and discharge of I^{131} in blocked slices of normal thyroid tissue.

At 100 μg. iodide per 100 ml., the total I^{127} uptake was 1.25 μg. I/gm. tissue. The I^{131} uptake in blocked slices was not altered by iodide between concentrations of from 0.2 to 100 μg. iodine per 100 ml. in the medium.

8. *Effect of Thiocyanate in Blocked Slices.* After a control period of incubation with 10^{-3} *M* methimazole, sodium thiocyanate was added at 10^{-2} and 10^{-3} *M* concentration. Figure 4 shows that thiocyanate discharged some, but not all, of the accumulated radioiodine, the discharge being more rapid at the higher concentration of thiocyanate than at the lower concentration. The iodide T/M ratios were 1.0 and 5.2 respectively for the thiocyanate-treated and control slices.

9. *Protein Binding of I^{131} in the Gravity-flow System.* The rate of protein binding in normal thyroid slices was estimated by terminating the incubation of slices at 30, 60, 90 and 180 minutes. In collateral experiments, thiocyanate (10^{-2} *M*) was introduced into different tissue chambers after 30, 60, 90 and 180 minutes of incubation with the standard medium in order to determine the quantity of dischargeable I^{131} present. It was apparent (Table 4) that the proportion of trichloroacetic-acid-precipitable I^{131} increased with the duration of incubation. Conversely, the portion of the total I^{131} in the tissue dischargeable by thiocyanate progressively decreased with time, being 50% at 30 minutes of incubation and 18% at 180 minutes of incubation.

TABLE 4. PROTEIN BINDING OF I^{131} AND DURATION OF INCUBATION

Duration of incubation, minutes	Trichloroacetic-acid-precipitable I^{131}, per cent		
	Experiment 1	Experiment 2	Experiment 3
30	32	20	34
60	42	28	42
90	28	55	44
180	56	64	66

COMMENT

The gravity-flow method makes it possible for thyroid slices to accumulate radioiodine as a simple linear function of time. This has obvious advantages, circumventing as it does some of the dynamic complexities of iodine metabolism as studied by tracer technics. These advantages, and those which stem from a method permitting experimental and control observations to be made on the same slice, may outweigh, at least in some situations, shortcomings inherent in an *in vitro* procedure. Moreover, by an *in vitro* system quantitative comparisons of various aspects of iodine metabolism of human thyroids can be made which are impossible by *in vivo* systems.

The linear rate of radioiodine accumulation with the gravity-flow system is probably a resultant of at least two concomitant processes: (1) a steady

absorption of radioiodide by the slice, and (2) a steady loss of radioiodide from the slice. The former process represents, at least in unblocked tissue, the sum of both the trapping and the protein binding of iodine. The latter process represents, at least in part, a loss of inorganic iodine and, probably to a substantial extent also, early loss of organic iodine from the slice.

The uptake of radioiodine by unblocked slices was inversely proportional to the inorganic I^{127} concentration. Two micrograms of inorganic I^{127} per 100 ml., the lowest concentration of stable carrier iodide which was tested, was found to depress radioiodine uptake slightly, but the depression was significant when compared with values observed using carrier-free solutions. It is of interest to compare this value (2 μg. per 100 ml.) with the value of 5 μg. inorganic I^{127} per 100 ml. observed *in vivo* by Stanley (1949) and by Childs *et al.* (1950) to depress the biosynthesis of thyroid hormone in hyperthyroid patients.

The accumulation of inorganic I^{127} by the blocked slice increased in exponential fashion with increasing concentration of inorganic iodide in the medium. Similar observations have been made *in vivo* (Leblond and Süe, 1941; Stanley and Astwood, 1948). In contrast with the effects observed in unblocked glands, increasing carrier I^{127} concentration had no observable effect on radioiodine accumulation by blocked slices. This is in agreement with previous observations *in vitro* (Freinkel and Ingbar, 1955; Schachner *et al.*, 1944) and *in vivo* studies (Halmi, 1954; VanderLaan and VanderLaan, 1947; Wyngaarden, Stanbury and Rapp, 1953; Childs *et al.*, 1950). The latter workers found it necessary to increase carrier I^{127} concentrations to a value of about 200 μg. per 100 ml. in order to reduce radioiodine accumulation in blocked tissue significantly.

The exponential pattern of radioiodine accumulation by the blocked thyroid gland could be distinguished in every experiment from the rectilinear pattern of uptake which was observed in unblocked slices. Inorganic iodine diffused slowly into the blocked thyroid gland, the half-value time being 215 ± 41 minutes at a flow rate of 4 ml./minute and 185 ± 55 minutes at a flow rate of 0.5 ml./minute. The rate for diffusion of inorganic iodide was slightly less than the rate of accumulation of radioiodine by the unblocked thyroid gland. This suggests that the rate of inorganic iodide diffusion ("trapping") may be a rate-limiting factor with regard to net rate of radioiodine accumulation by unblocked tissue.

The conditions under which radioiodine accumulation in both blocked and unblocked thyroid slices was examined in this study differ from the conditions in all previous studies (Schachner *et al.*, 1944; VanderLaan and Bissell, 1946; VanderLaan and Greer, 1950; VanderLaan and VanderLaan, 1947; Taurog, Chaikoff and Feller, 1947; Stanley and Astwood, 1948) in that slices of normal human thyroids were acutely exposed to constant concentration of blocking agent, radioiodine, and stable iodine. The experiments of Berson and Yalow (1955) and Ingbar (1955), in which radio-

iodide was administered intravenously following a blocking agent, perhaps most closely simulate our *in vitro* observations. Ingbar (1955) concluded that protein binding proceeds at a constant rate but that trapping or the accumulation of inorganic radioiodide proceeds at an exponential one.

The T/M ratio of 17.7 ± 3.9 compares well with the analogous thyroid/plasma ratio of 25 to 50 estimated for normal thyroid tissue *in vivo* (VanderLaan and VanderLaan, 1947; Stanley and Astwood, 1949), considering the obvious differences between *in vitro* and *in vivo* conditions. The action of thiocyanate also appears substantially the same *in vitro* as had previously been reported in numerous *in vivo* studies. The rapid rate of iodide discharge after thiocyanate was in marked contrast with its slow accumulation. The discharge of inorganic iodide by thiocyanate was always incomplete. As Raben (1949) has noted *in vivo*, the T/S (thyroid/serum) iodide ratio was not reduced below 1.

The proportion of radioactive iodine which was protein-bound in the slice was found to increase progressively with time. Similar observations *in vitro* were reported by Schachner *et al.* (1944) and Morton and Chaikoff (1943). In euthyroid human subjects, Ingbar (1955) estimated that 90% of the iodine contained within the thyroid iodide space was bound to protein each hour. Again, as anticipated, protein binding, as well as other thyroidal activities, was much slower *in vitro* than *in vivo*.

SUMMARY

A method, which we have termed "gravity-flow incubation," has been described for incubating human thyroid slices in a constantly flowing, nonrecirculating medium of constant specific activity. With this method the following were observed: 1. The pattern of accumulation of radioactive iodine by unblocked thyroid slices was rectilinear with respect to time. 2. The rate of radioiodine accumulation of thyroid slices was directly proportional to the rate at which the medium flowed through the tissue chamber. 3. The iodide uptake was decreased by inorganic iodine, L-triiodothyronine, L-thyroxine and thyroglobulin. 4. Under the influence of antithyroid drugs, the radioiodine accumulation appeared to be an exponential function and most but not all of the radioiodine of the blocked slice could be discharged by thiocyanate. 5. The proportion of radioactive iodine which was protein-bound increased with the duration of incubation of normal slices.

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ACTION OF ESTROGENS ON LACTIC DEHYDROGENASE-DPNH OXIDASE SYSTEM OF RAT UTERUS

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THE vital role of the enzyme system which catalyzes the oxidation of lactate and reduction of pyruvate in the final reductive phase of glycolysis has made an investigation of this enzyme system in the uterus seem worthwhile from the point of view of the possible influences which steroid hormones may have upon it. Recent investigations of Telfer (1952) and Longwell and Reif (1954) have shown an increase in uterine succinoxidase and cytochrome oxidase in ovariectomized rats following estrogen injections. Hagerman and Villee (1953) have reported estradiol to increase the oxygen consumption of human endometrium *in vitro* and to shift the metabolism of glucose and pyruvic acid to more complete oxidation. In more recent reports, Villee, Loring and Wellington (1954) and Villee (1954), (1955) have indicated an *in vitro* site of action in the Krebs tricarboxylic acid cycle localized between citrate utilization and α-ketoglutarate production. Although other enzymes may be involved, they were able to show a direct *in vitro* stimulation by estradiol of a diphosphopyridine nucleotide (DPN) linked isocitric dehydrogenase of human endometrium and term placenta. Lucas and co-workers have very recently (1955) reported an *in vitro* increase in uterine peroxidase following estrogen treatment of ovariectomized rats.

Estrogens also seem to differ in their ability to promote growth of the uterus in rats. Such comparisons have been made for estradiol, estrone and estriol by Hisaw, Velardo and Goolsby (1954) who also report competitive interactions between these estrogens. In the present paper the effects of these naturally occurring estrogens upon the lactate oxidizing system of uteri from ovariectomized rats have been studied in order to provide further information upon the manner in which estrogens exert and effect upon uterine oxidative metabolism.[4]

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[4] A preliminary report of this work was presented at the annual meeting of the Federation of Americal Societies for Experimental Biology, Chicago, April, 1953. (Bever, Velardo and Hisaw, 1953a).

The lactate oxidizing system of animal tissues as described by Corran, Green and Straub (1949), consists of the specific protein lactic dehydrogenase (LDH) with its coenzyme, diphosphopyridine nucleotide (DPN), and a flavin enzyme of diaphorase or DPN-cytochrome *c* reductase nature. This system, either alone or in combination with other carriers, transfers electrons into the cytochrome system and subsequently to oxygen. In the test system utilized by Straub (1940), a redox dye is substituted for the cytochrome components and the dye is reduced directly by the flavoprotein. In order to avoid an implied identification of the uterine flavin enzyme with either diaphorase or DPN-cytochrome *c* reductase, terms currently applied to well-characterized flavin enzymes of heart, liver and skeletal muscle, we shall refer to the uterine enzyme as DPNH oxidase, since, in activity measurements by redox dyes, the physiological electron acceptor is undefined. The system will then be referred to as the LDH-DPNH oxidase system. Straub (1940) used methylene blue as the acceptor dye; however, since the recent introduction of the tetrazolium dyes as the acceptor for dehydrogenating enzymes, these have become popular (Smith 1951). One distinct advantage is that a water-insoluble product is formed which is not reversibly oxidized by oxygen as is methylene blue. Tetrazolium dyes have been found useful for specifically measuring dehydrogenase-diaphorase systems (Brodie and Gots, 1951).

EXPERIMENTAL

A. *The assay method for LDH-DPNH oxidase in rat uteri*

The rats were killed by decapitation, the uteri rapidly excised and the luminal fluid expressed. The uteri were quickly weighed and placed in tubes in a bath of crushed ice. The horns of each uterus were divided at the cervix. One horn was used for the determination of water content by oven-drying for 24 hours at 90–100° C. Nitrogen determinations were made upon the dried, ground uteri by nesslerization after digestion in selenium sulfuric acid mixture. The other horn of each uterus was homogenized in a Potter-Elvehjem homogenizer in an ice bath with 12 ml. of cold 0.067 *M* phosphate buffer containing 0.1% NaCN and 0.1% nicotinamide at pH 8.2. Alternatively, three to five individual uterine horns of a group of animals were homogenized together in 16–20 ml. of buffer. The average activity of individually processed horns and that of pooled horns agreed very closely. One ml. aliquots of the homogenate were placed in 15 ml. graduated centrifuge tubes containing 0.5 ml. of sodium lactate (500 μ moles) and 0.3 ml. of 0.1% DPN solution (0.453 μ mole). Substrate blanks without lactate but containing equivalent distilled water were also prepared. Triplicate "test" tubes and single "blank" tubes were prepared for each uterus or group of uteri assayed. Each tube was brought to a volume of 2.0 ml. with buffer. When all tubes of a test series were prepared, 0.5 ml. of 0.4% neotetrazolium chloride (NTZ)[5] (3.75 μ moles) was rapidly pipetted into each tube and all tubes of the test were incubated for 30 minutes at 34° C. Aliquots of each tissue homogenate tested, as well as aliquots of the buffer described, were dried at 90–100° C for 24 hours. Both the homogenate and buffer samples were at 2° C $\pm$ 1° C when sampled for the dry weight determinations.

[5] NTZ was obtained from Montclair Research Corporation, Montclair, N.J., and was found suitable for use without further purification.

The enzymatic reaction was stopped by adding 1.0 ml. of 10% trichloracetic acid to each tube. The tubes were then centrifuged in the cold and the clear supernatant discarded. The protein residue containing the violet-colored reduced NTZ was extracted thoroughly with 10 ml. of chilled acetone and again centrifuged in the cold to remove protein. Centrifugation at room temperature resulted in marked discoloration in the blank tubes. The supernatant fractions were cleared of haziness due to insoluble phosphate by the addition of 0.2 ml. of 0.1 N HCl to all tubes. The optical density of each tube was obtained by colorimetric determination in a Klett-Summerson colorimeter using the green number 54 filter. The reading for each tube was converted to μg. of

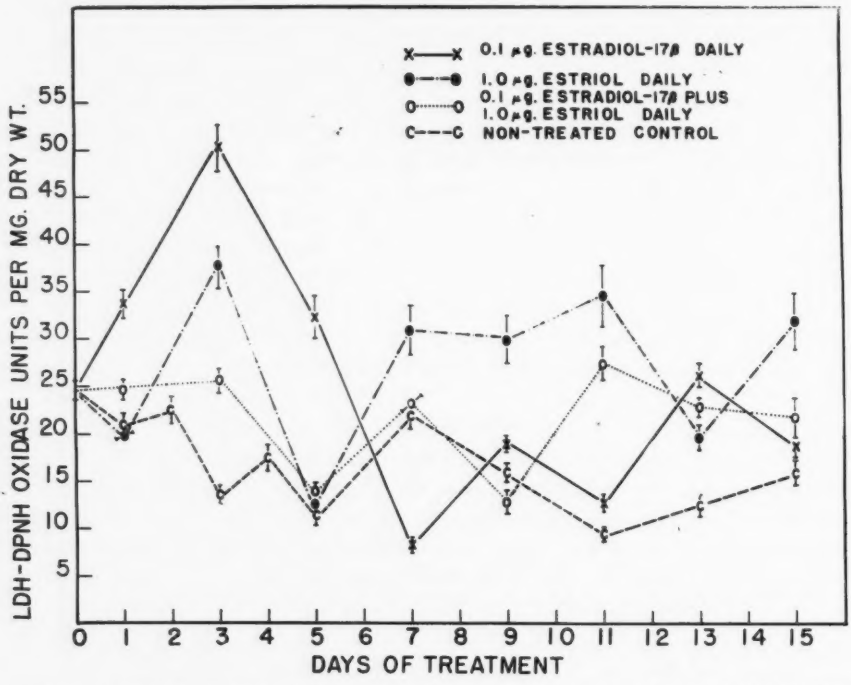

FIG. 1. The effect of estradiol and estriol on ovariectomized rat uterine LDH-DPNH oxidase system as a function of time. Each point represents a minimum of 12 determinations of activity from 4 animals run in triplicate. The standard error of the mean for each point is indicated by vertical lines.

reduced NTZ by multiplying the optical density by an empirical constant obtained from a standard curve of NTZ reduced by sodium hydrosulfite ($Na_2S_2O_4$) and measured similarly.[6] Twenty to 200 μg. NTZ in 1.0 ml. of water were used in the preparation of the standard curve. One-tenth ml. of 50 mg.% sodium hydrosulfite in freshly prepared 1% $NaHCO_3$ was used to reduce each tube of the standard series. After reduction they were diluted to 10 ml. with cold acetone.

Under the conditions given, the protein lactic apodehydrogenase and the flavin enzyme, DPNH oxidase, form a system which is rate-limiting with other components in

[6] The constant employed for our colorimeter for these experiments was: K = 17.0 Klett density units/μgm. NTZ reduced/ml. acetone.

excess. As a result, the term μg. of NTZ reduced per mg. of dry tissue, under standard conditions of time, temperature and pH serves as a unit of activity of the LDH-DPNH oxidase system and for each of its components when measured individually. The observed rate between 15 and 40 minutes is linear with time and, under these conditions, is also linear with tissue concentration over a ten-fold range. Under most experimental conditions imposed upon the uterus, its weight is such that the tissue concentration as described would be in the middle third of this range. Although nitrogen determinations were made upon all uteri reported, the expression of activity for this enzyme system is experimentally as meaningful on a dry weight basis as on a nitrogen basis since on these experiments dry weight and total uterine nitrogen parallel one another precisely.

Since either of the enzymes in the system may be the limiting step, experiments were designed in which data for the activity of the individual LDH and DPNH oxidase components were obtained. Such data were obtained in all experiments except those furnishing data for Figure 1 and Table 1. Separate LDH and DPNH oxidase activities were determined by preparing extra incubation tubes in the manner described above

TABLE 1

Treatment	Total area (unit-days)	Total area minus control area	% of response to 0.1 μg. estradiol
1. 0.1 μg. Estradiol	377	138	100
2. 1.0 μg. Estradiol	408	169	122
3. 0.1 μg. Estradiol plus 1.0 μg. Estriol	323	84	61
4. 0.05 μg. Estradiol	313	74	54
5. Control	239	—	—

for the complete LDH-DPNH oxidase system; additions of heart muscle LDH in excess were made in triplicate tubes to make the uterine flavoprotein (DPNH oxidase) the limiting step, and the addition of an excess of heart muscle diaphorase was made to three other tubes to make the uterine LDH the limiting step. These tubes were incubated and the color developed in the same manner as described above for the LDH-DPNH oxidase system.

Heart muscle LDH was prepared by lyophilization of the preparation described by Sumner and Krishnan (1948). Five mg. of the LDH powder were added to each reaction tube in 0.2 ml. of 0.067 *M* phosphate buffer, pH 8.2. The insoluble beef heart diaphorase preparation described by the same authors was lyophilized, ground to a fine powder, ether extracted once and again ground to a fine powder. One-half gram of the powder was extracted with 10 ml. of 0.067 *M* phosphate buffer, pH 8.2, for 5 minutes at 40° C and centrifuged. Two tenths ml. of the supernatant was sufficient to provide excess of diaphorase. Neither the LDH nor diaphorase preparation contained any activity of the other.

B. *Estrogen Treatments*

All animals used in these experiments were 100 day old albino rats of the Harvard colony with a body weight of 200 ± 10 grams at the time of ovariectomy. The animals were placed on experiment seven days after castration. Estrogens[7] were injected subcutaneously once a day in 0.1 ml. of sesame oil. When two estrogens were administered concurrently they were injected separately at different sites.

[7] The estrogens used in these experiments were obtained through the generosity of Schering Corporation and of Parke, Davis and Company.

1. *15 Day Injection Period.* Groups of 5–7 ovariectomized animals were injected daily with 0.05μg. estradiol-17β, 0.1 μg. estradiol-17β, 1.0 μg. estriol and with a combination dosage of 0.1 μg. estradiol-17β plus 1.0 μg. estriol. Groups in each dosage regimen were killed at one or two day intervals up to 15 days (Fig. 1). Control groups receiving no treatment were simultaneously placed on experiment for the same periods. The treated and control groups were killed at the appropriate time intervals and the uterine LDH-DPNH oxidase system activity determined. The results are recorded

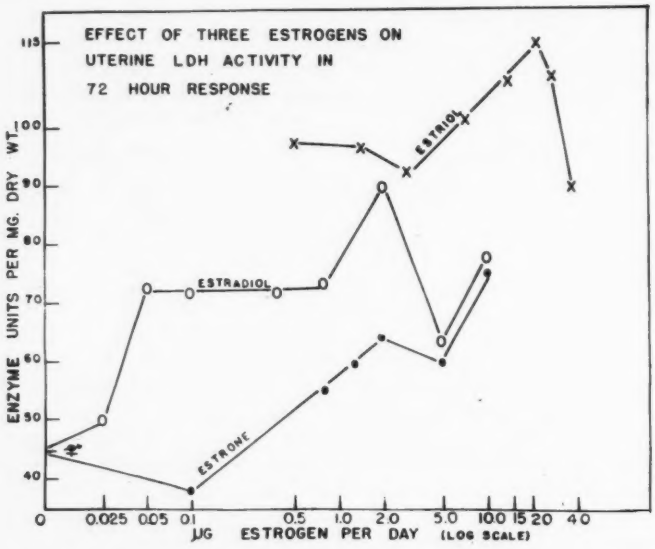
EFFECT OF THREE ESTROGENS ON
UTERINE LDH ACTIVITY IN
72 HOUR RESPONSE
ENZYME UNITS PER MG. DRY WT.
ESTRIOL
ESTRADIOL
ESTRONE
0 0.025 0.05 0.1 0.5 1.0 2.0 5.0 10.0 15 20 40
μG ESTROGEN PER DAY (LOG SCALE)

FIG. 2. Effect of three estrogens on uterine LDH activity in 72 hours. Each point represents the average of triplicate determinations from 4–5 pooled uterine horns.

in Figure 1 and in Table 1. The activity for 0.05 μg. estradiol was not recorded on Figure 1 to avoid confusion caused by too many lines. The summated response for the 15 day period is approximately half that of 0.1 μg. estradiol (Table 1).

2. *72 Hour Dose-Response Curves for Three Estrogens.* Estradiol-17β, estrone and estriol in graded amounts were injected daily for three days and the animals killed 72 hours after the first injection. The dosages ranged from a lower limit of 0.025 μg. of estradiol to 37.5 μg. of estriol. Groups of 4–5 animals were used for each dosage. The uterine LDH and DPNH oxidase activities were assayed for each of the dosage groups (Figs. 2, 3, 4).

RESULTS

When a dosage of 0.1 μg. estradiol per day is given to castrated rats for as long as fifteen days, the uterine LDH-DPNH oxidase activity rises to a

maximum on the third day of treatment (Fig. 1). On the third day (72 hr.) the uteri receiving 0.1 μg. estradiol are increased in specific enzyme activity (μg. reduced NTZ/mg. dry wt.) 3.72 times that of the corresponding castrate control group (50.2 ÷ 13.5 units). In previously reported results of the uterine weight changes occurring in this experiment (Hisaw, Velardo and Goolsby, 1954) the corresponding dry weight was increased by a factor of 2.9 (39.5 ÷ 13.6 mg.). Thus the total enzyme activity can be calculated to increase 10.8 times (1980 ÷ 183 units). There is a marked fall from this peak and a minumum of activity is reached at seven days which

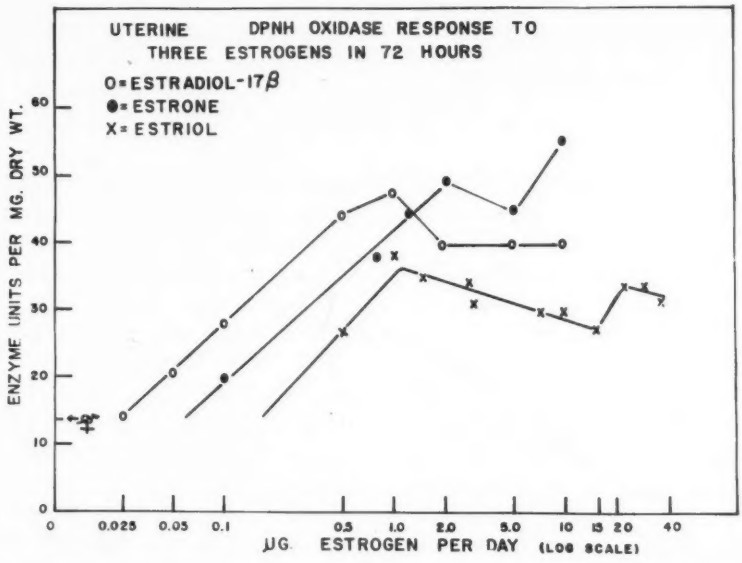

FIG. 3. Uterine DPNH oxidase response to three estrogens in 72 hours. Each point represents the average of triplicate determinations from 4–5 pooled uterine hrons.

is below the corresponding control value. When 0.05 μg. per day is given (not shown in Fig. 1), the earliest peak response is reached on the fifth day (27.1 units), followed by a recovery by day 15 (28.3 units) equivalent to the earlier three day response. The data suggest that under continued estrogen injection an opposing reaction to the stimulatory effect is encountered on or about the third day.

Although the enzyme system under the influence of daily injections of 0.1 μg. estradiol reaches a greater response at 72 hours than when 1.0 μg. estriol is given (Fig. 1), the latter treatment produces a more rapid decrease in enzyme activity immediately after 72 hours. The recovery of estriol-treated uteri is more rapid and regains a greater activity than with 0.1 μg. estradiol. When the two estrogens are given concurrently at their respective dosages, the resulting response in the enzyme system is markedly less than

that produced by either steroid alone and is not significantly greater than the untreated control groups until after day nine.

A comparative evaluation of the effects of these steroid treatments upon the enzyme system was obtained by determining the areas under their respective curves. These measurements were expressed in terms of enzyme unit-days and their values compared percentagewise with the response to 0.1 μg. estradiol (Table 1). Thus, the highest integrated response over

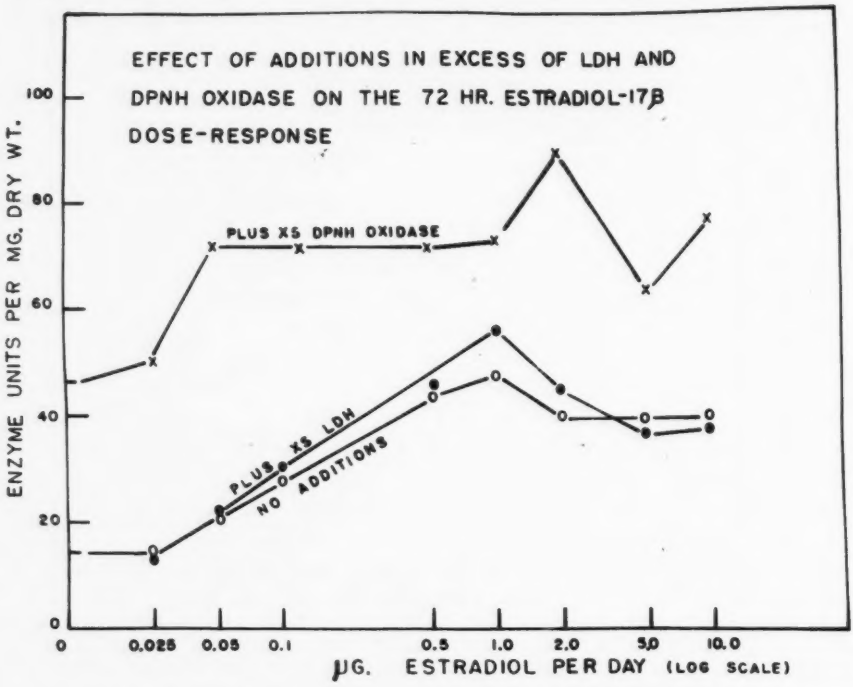

FIG. 4. The effect of additions in excess of LDH and DPNH oxidase on the 72 hour estradiol-17β dose-response. Four pooled uterine horns were used to prepare the homogenates. Each point represents the average of triplicate determinations.

the same period was achieved by 1.0 μg. estriol daily, although 0.1 μg. estradiol produced the highest initial response at three days. When the estradiol and estriol dosages were administered concurrently, the effect was strikingly less than that for either dosage alone. The reaction to the combined dosage of the two hormones over the 15 day period was approximately equivalent to that produced by 0.05 μg. of estradiol when administered alone.

These experiments show that estradiol, estriol, and the two hormones combined, produce characteristic response curves when given in the

amounts shown in Figure 1 for a period of 15 days, and also that a maximal effect is attained on the third day. Therefore a period of three days was chosen as a suitable length of treatment for comparing the effects of graded doses of the three estrogens on the LDH-DPNH oxidase system and the two individual enzyme activities.

Estriol appears to be able to raise LDH activity to a higher level than either estradiol or estrone in the 72 hour test (Fig. 2). The maximum LDH response to estriol in three days is produced by 22.5 μg. daily, while the maximum for estradiol is at 2.0 μg. The response to estradiol seems to be approximately the same for doses between 0.05 and 1.0 μg. daily. A definite peak response for estrone was not reached in these experiments. Although all three estrogens exhibit an apparent self-inhibition in the dose range of 2–5 μg., estriol produces a maximum response in LDH activity with a daily dosage of 22.5 μg., followed by a rapid drop in activity at higher values.

While LDH can be promoted to a higher activity with estriol, this hormone is the least effective of the three in promoting activity of the DPNH oxidase component of the enzyme system (Fig. 3). Estradiol and estrone promote this part of the system to a greater extent than estriol. Up to 1.0 μg. daily, estradiol produces a greater DPNH oxidase response then does estrone. Above this, estrone is more potent. Both estriol and estradiol reach a maximum response at a dosage of 1.0 μg. daily while again, as with LDH, estrone shows no clear cut maximum with this dose range.

The experiments designed to test the relative importance of LDH and DPNH oxidase as limiting factors in the enzyme system following an estrogen treatment indicate that changes in activity of the flavoprotein are chiefly responsible for the extent of the reaction (Fig. 4). In fact, when the LDH-DPNH oxidase system of a uterine homogenate is measured, the resulting activity is essentially the same as the specific measurements of DPNH oxidase. Thus, under these conditions, the DPNH oxidase component is the limiting step of the system as measured *in vitro*, since the addition of an excess of heart diaphorase in the determination of LDH in our experiments has invariably resulted in higher activity of the two enzyme system. Also the dose-response curve for the LDH has a different shape from that for either DPNH oxidase or the endogenous LDH-DPNH oxidase system. Conversely, the addition of excess heart LDH in the measurement of uterine DPNH oxidase does not increase the oxidation rate of lactate in any functional state of the rat uterus investigated. The dependence of rate of action on DPNH oxidase apparently is not confined to this method of measurement. DPNH oxidase can be shown to be the limiting step when either uterine homogenates or slices are used in Warburg respiration studies with oxygen as acceptor from the cytochrome system, or from methylene blue.

The rate-limitation by DPNH oxidase as measured *in vitro* seems to be true of the rat uterus under all conditions studied to date. It was found after injection of several different types of steroids into castrated animals, and was found to obtain in all phases of the normal estrous cycle (Bever, Velardo, Telfer, Hisaw and Goolsby, 1954).

DISCUSSION

Attempts to show an activation of the LDH-DPNH oxidase system by *in vitro* addition of estradiol in amounts of 0.01 to 10 μg. per ml. of homogenate showed no effect on the system. Incubation times varied from 0.5 to 3 hours. Similar experiments with intact uterine horns incubated for periods between 1 and 6 hours before homogenization and assay showed no increase in activity over comparable control horns. To the extent that such negative data may be indicative, the results support the viewpoint that the site of activation of this enzyme is at a secondary level, perhaps in increasing specific protein synthesis of the enzymes involved, rather than a direct activation of the existing enzyme molecules.

Since substrate level and coenzyme level are in excess in the assay, the enzyme protein has apparently increased due to the physiological action of the injected steroids. However, the total increase in the specific proteins of these enzymes, based on activity, is greater than the simultaneous increase in total uterine protein by a factor of 3–4 for a dosage of estradiol producing maximum enzyme response. The fresh and dry weight data of the uteri under treatment in this paper have been reported previously by Hisaw, Velardo and Goolsby (1954). These latter data in the 15-day experiment show no large scale fluctuations during the period, in sharp contrast to the present enzyme data, indicating that the periodic loss of enzyme activity under continued estrogen injection is related to specific protein rather than general protein.

When an enzyme is increased in activity by hormone action, one of the possible sites of control is upon the coenzyme or prosthetic group of the enzyme, i.e., by making more coenzyme or prosthetic group available where it is otherwise limiting. In the case of DPNH oxidase, the increased activity is not due to an increase of prosthetic group since additions of either flavin adenine dinucleotide or riboflavin phosphate do not increase the DPNH oxidase activity of uteri of castrated, castrated and estrogen injected, or normal intact rats in diestrus or estrus. Since DPN, the coenzyme for LDH, was added in excess to the incubation mixture, and LDH changes were observed after estrogen was administered, it seems unlikely that the estrogen action observed here is at this coenzyme level. Nevertheless, it is interesting to note that the enzyme system as measured exhibited an absolute requirement for exogenous DPN. If no DPN were added, no tetrazolium reduction occurred in the presence of lactate even in those homogenates prepared from estrogen treated uteri. Attempts to estimate

endogenous uterine DPN content failed with methods adequate for determination of DPN content of liver, kidney and skeletal muscle. Either the co-enzyme is in very low concentration or is rapidly destroyed after homogenization in buffer containing nicotinamide. The low concentration of uterine DPN has previously been commented upon by Hollman (1949). We have not, in these experiments, eliminated DPN concentration as a mechanism of estrogen control.

The 15 day experiments involving estradiol and estriol measured the activity of the homogenate LDH-DPNH oxidase system. In view of the data in Figure 4 and evidence that DPNH oxidase is the limiting step in all subsequent treatments and in intact rats, it seems probable that the fluctuations of the two enzyme system under constant daily hormone treatment reflects DPNH oxidase changes rather than LDH.

We have no explanation as yet for the high amplitude flucations of the enzyme system during the constant estrogen treatments in the 15-day experiments. However, it has been mentioned in a preliminary report (Bever, Velardo and Hisaw, 1953b) that desoxycorticosterone acetate and cortisone acetate inhibit estradiol in the activation of uterine DPNH oxidase. This would indicate the possibility that the adrenal may be involved in the fluctuating enzyme response to constant estrogen treatment.

The biochemical studies were supplemented by histochemical localization in the uterine tissues in which increased activity of the LDH-DPNH oxidase enzyme system occurred. The histochemical technique used was the neotetrazolium reaction preliminarily reported by Rosa (1953) and to be published in detail elsewhere (Rosa and Bever). The areas showing LDH-DPNH oxidase activity responsive to estrogen were the luminal epithelium and glands and the myometrium. However, the longitudinal musculature appeared to produce a more intense reaction than did the circular layers, but the highest concentration of enzyme was in the luminal epithelium. The stroma of the endometrium showed little enzyme activity even after estrogen treatment.

SUMMARY

Estradiol-17β, estrone and estriol have been shown to increase significantly the specific activity of the lactic dehydrogenase-DPNH oxidase system in uteri of ovariectomized rats. Both enzymes of the system are increased, but to different degrees and in a different order of effectiveness by the three steroids. Estradiol is more effective and estriol least in increasing DPNH oxidase, while estriol is more potent in increasing the LDH component and estrone least. Under the experimental conditions employed, DPNH oxidase was demonstrated to be the rate limiting step in the system. The daily uterine enzyme levels produced by constant daily estrogen doses to ovariectomized rats fluctuated during a 15-day period in a manner suggesting an opposing secondary control by unknown factors.

During this period, the level of enzyme system activity produced by estradiol and estriol given simultaneously was less than that achieved by either estrogen when given alone.

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THE ENDOCRINE SOCIETY

The 1956 Annual Meeting

The Thirty-eighth Annual Meeting of The Endocrine Society will be held in the Palmer House, Chicago, Illinois, Thursday, Friday, and Saturday, June 7–8–9, 1956.

The Committee on Local Arrangements is Dr. Norris J. Heckel as Chairman with Drs. M. Edward Davis, Sam Taylor, J. P. Greenhill, and Harley E. Cluxton as members of the Committee.

All Scientific Sessions will be held in the Palmer House. The rooms in which each session will be held will be announced in the program and on the hotel bulletin board. The Annual Dinner is scheduled for Friday, June 8, at 7:30 p.m., preceded by cocktails at 6:30 p.m.

All members are urged to make hotel reservations immediately inasmuch as the hotels expect to be filled to capacity. Make reservations directly with the Palmer House, advising time of arrival and departure date. Make your reservations now and avoid disappointment.

The Council requests that members send suggestions for nominations for the elective offices of the Society to the Secretary. Those desiring to do so should send this information to the Secretary immediately for transmission to the Nominating Committee.

AWARDS OF THE ENDOCRINE SOCIETY

The selection of the recipients of the awards of The Endocrine Society is made by a Committee appointed by the Council of the Society. These awards and fellowships carry no obligation by the recipient to the Society or to the donors.

Medal of The Endocrine Society

In 1954 the Council of the Society voted to establish a medal and an honorarium of $1,000 to be given to an individual for work of special distinction in endocrinology. The recipient shall be chosen from nominations presented by members of the Society and is limited to citizens of the United States and Canada. In 1955 the Medal was presented to Dr. Carl R. Moore.

The Ciba Award

The Ciba Award to recognize the meritorious accomplishments of an investigator not more than 35 years of age in the field of clinical or preclinical endocrinology was established in 1942, but no recipient was selected in 1942 or 1943. In 1944 the Award was presented to Dr. E. B. Astwood; 1945, Dr. Jane A. Russell; 1946, Dr. Martin M. Hoffman; 1947, Dr. Choh Hao Li; 1948, Dr. Carl Heller; 1949, Dr. George Sayers; 1950, Dr. Oscar M. Hechter; 1951, Dr. Albert Segaloff; 1952, Dr. Seymour Lieberman; 1953, Dr. Sidney Roberts and Dr. Claire Szego (Mrs. Roberts); 1954, Dr. Isodore M. Rosenberg; 1955, Dr. Jack Gross. Prior to 1952 the Award was $1,200. It is now $1,800. If within twenty-fourth months of the date of the Award, the recipient should choose to use it toward further study in a laboratory other than that in which he is at present working, it will be increased to $2,500.

The Ayerst, McKenna and Harrison Fellowship

This fellowship was established in 1947. It is designed to assist men or women of exceptional promise in furthering their advancement towards a career in endocrinology. The fellowship is awarded on alternate years (1955, 1957, etc.) and the stipend which will not exceed $5,000 may be divided into two Fellowships in varying amounts in accordance with the qualifications of the appointees. Individuals possessing the M.D. or Ph.D. degree or candidates for either of these degrees are eligible for appointment.

Applicants must submit the following information:

(1) Evidence of scientific ability as attested by studies completed or in progress.

(2) Recommendations from individuals familiar with the candidate and his work.
(3) A proposed program of study.
(4) Acceptance of the individual by the head of the department in which the Fellowship will be held.
(5) A statement that he or she will serve full time if awarded a Fellowship. A small amount of time (10 to 15 per cent) may be spent in course work or participation in teaching, the latter purely on a voluntary basis.

The Schering and The Upjohn Scholars of The Endocrine Society

The Council of The Endocrine Society established a category of Scholars. These grants have been made available through the generosity of The Schering Corporation and The Upjohn Company, and will be awarded to established investigators and teachers in the fields of endocrinology who wish to extend their opportunities for work either in this country or abroad.

The award will not exceed $2,500 annually for each individual, and shall be granted on the basis of proposals submitted by the applicant and such applications should include the estimated financial needs. The funds may be used for travel, maintenance and other expenses.

Nominations

Nominations for the Medal of The Endocrine Society; the Ciba Award; and the Ayerst, McKenna and Harrison Fellowship may be made by any member of The Endocrine Society. They should be submitted on forms which may be obtained from the Office of the Secretary, 1200 N. Walker Street, Oklahoma City 3, Oklahoma. Completed nominations should be returned to the Secretary not later than November 1st each year.

Proposals for appointment as Scholars of The Endocrine Society should be made in writing by the individual, and addressed to the Secretary of the Society. They should be submitted by November 1, 1955.

The Awards Committee will meet in November and notice of awards to successful nominees and applicants will be made not later than December 1, 1955.

THE EIGHTH POSTGRADUATE ASSEMBLY IN ENDOCRINOLOGY AND METABOLISM

Sponsored jointly by the Endocrine Society; The University of Texas Postgraduate School of Medicine, and the University of Texas M. D. Anderson Hospital and Tumor Institute,
Houston, Texas.
October 2–27, 1956

The faculty will consist of twenty-two eminent clinicians and investigators from various parts of the country in the fields of endocrinology and metabolism. The program will cover the various endocrinopathies, with emphasis on the clinical aspects, demonstration of laboratory tests, presentations of cases, and question-and-answer panel discussions. The course is designed to cover the main aspects of diagnosis and therapy in the field of endocrinology and metabolism for the physician in general practice and for those in other specialties who wish to have a general knowledge of this rapidly growing field.

A syllabus with brief abstracts of lectures will be available to the registrants at the time of the assembly.

For further information concerning the program and registration, write to Dr. J. B. Trunnel, M. D. Anderson Hospital, 6723 Bertner Avenue, Houston 25, Texas. Registration is limited to 100; tuition fee is $100.00. Rooms will be reserved for the students and faculty at the Shamrock Hilton Hotel which is adjacent to the Texas Medical Center.

THE BRITISH SOCIETY FOR ENDOCRINOLOGY

Memoirs of the Society for Endocrinoloy

The attention of members of The Endocrine Society is drawn to the publication of Memoirs No. 4 and 5 of the Society for Endocrinology reporting the proceedings of a Symposium on The Comparative Physiology of Reproduction held last year at Liverpool University.

Memoir 4 reviews reproductive patterns in fish, amphibia, reptiles and mammals, and includes accounts of the evolution of viviparity and vertebrate gonadotrophins. The volume comprises 265 pages, numerous illustrations and one colour plate.

Memoir 5 reports the second part of the Symposium under the title The Hormonal Control of Water and Salt-Electrolyte Metabolism in Vertebrates. It consists of about 140 pages and many text figures, and gives a comprehensive review of the endocrine control of water and salt-electrolyte metabolism in different vertebrates.

Copies may be obtained at a cost of 50/- and 25/- respectively through booksellers, or from the Cambridge University Press, Bentley House, London, N.W. 1.

In the U.S.A. copies may be obtained at a price of $8.50 and $4.75 respectively through booksellers or from the New York: Cambridge University Press, 32 East 57th Street, New York 22.

NEW BOOK REGISTER

PRACTICAL ENDOCRINOLOGY. Lewis M. Hurxthal in cooperation with A. Seymour Parker and Hirsh Sulkowitch. A handbook for the general practitioner. New York, N. Y.: McGraw-Hill Book Co., Inc., 1955. 318 pages. $7.00

THE TECHNIQUE AND SIGNIFICANCE OF OESTROGEN DETERMINATIONS. P. Eckstein and S. Zuckerman. Proceedings of a conference held jointly by the Society of Endocrinology and the Endocrinological Section of the Royal Society of Medicine at the Royal Society of Medicine, London, on 17 February, 1954. Cambridge, England: Cambridge University Press, 1955. 94 pages. $3.75

CLINICAL ENDOCRINOLOGY. Second Edition. Laurence Martin and Martin Hynes. A practical account of endocrine disease from the viewpoint of the general physician, together with the essential details of physiological and pathological background. Boston, Massachusetts: Little, Brown & Company, 1955. 253 pages. $5.50

1955 MEDICAL PROGRESS. Morris Fishbein, editor. A review of medical advances during 1954. New York, N. Y.: The Blakiston Division, McGraw-Hill Book Company, Inc., 1955. 346 pages. $5.00

HYPEROSTOSIS CRANII. Sherwood Moore. The history of the disorder is given in full with a chronologically arranged bibliography. Springfield, Illinois: Charles C Thomas, Publisher, 1955. 226 pages. $10.00

THE THYROID. In this, the seventh annual symposium of the Biology Department of Brookhaven National Laboratory, an attempt was made to collate and discuss findings and ideas relating to problems of the thyroid and thyroid hormone physiology. Available at the Office of Technical Services, Department of Commerce, Washington 25, D. C. $1.75

LIFE STRESS AND ESSENTIAL HYPERTENSION. Stewart Wolf, Philippe V. Cardon, Jr., Edward M. Shepard and Harold G. Wolff. A study of circulatory adjustments in man. Baltimore: The Williams & Wilkins Company, 1955. 253 pages. $7.50

BIOCHEMISTRY AND THE CENTRAL NERVOUS SYSTEM. Henry McIlwain. The story of chemical change and structure in the central nervous system presents to the biochemist a well-developed branch of his own subject and one which has often been in the forefront of biochemical discovery. Boston: Little, Brown and Company, 1955. 272 pages. $9.50.

GENERAL ENDOCRINOLOGY. C. Donnell Turner. Second Edition. Much of the material originally contained in Chapters 1 and 2 has been condensed and placed in a new chapter near the end of the book where it is more likely to be understood by the beginning student. The final chapter on Hormones in the Invertebrates has been condensed and brought up to date. In view of the tremendous amount of research published since 1948 on the adrenal cortex and pituitary gland, these sections have been practically rewritten. All chapters have been revised thoroughly and brought up to date. The sections on biochemistry have been expanded and placed near the beginning of each chapter; the sequence is now anatomy, biochemistry and physiology. A new chapter has been added on adaptive reactions to stress. Philadelphia, Pennsylvania and London: W. B. Saunders, 1955. 553 pages. $13.00

PSYCHIATRIC RESEARCH REPORTS of the American Psychiatric Association. Edited by Members of the Committee on Research, 1954–55. Pharmacologic products recently introduced in the treatment of psychiatric disorders. Orders should be addressed to: Psychiatric Research Reports, American Psychiatric Association, 1785 Massachusetts Avenue, N.W., Washington 6, D. C. $2.00